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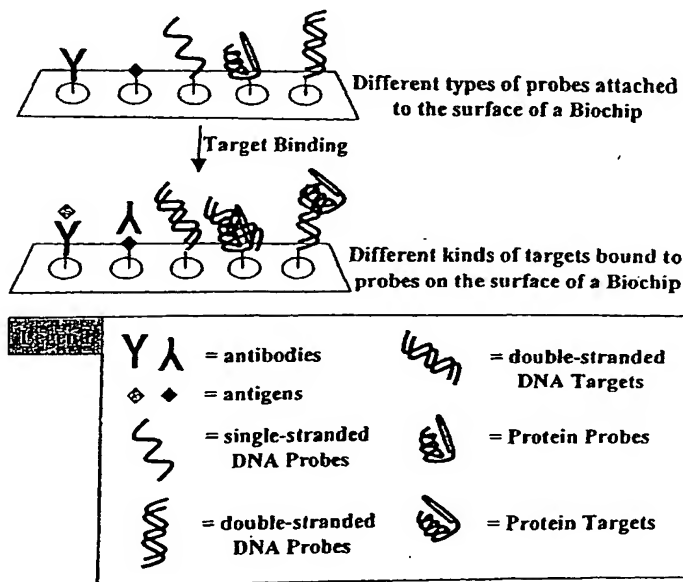
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(54) Title: METHODS AND DEVICES FOR DETECTING HYDROCARBONS AND PETROLEUM PRODUCTS



(57) Abstract: The invention relates to methods and devices for detecting hydrocarbons, such as petroleum, in samples, such as environmental samples, e.g., soil, sand, and water.

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## METHODS AND DEVICES FOR DETECTING HYDROCARBONS

### CROSS REFERENCE TO RELATED APPLICATIONS

This application claims priority from U.S. Provisional Patent Application Serial No. 60/308,870, filed on July 30, 2001, and U.S. Patent Application Serial No. 09/996,056, filed on November 27, 2001, which are both incorporated herein by reference in their entirety.

### TECHNICAL FIELD

This invention relates to methods and devices for detecting hydrocarbons such as petroleum in samples such as soil and water samples.

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### BACKGROUND

Geochemical petroleum prospecting typically relies on the collection of soils, sediments, or rocks and the subsequent analysis of these media for materials associated with petroleum, such as specific hydrocarbons that make up petroleum. Some of these hydrocarbons are either highly toxic to microorganisms, or may be used by the microorganisms as a source of carbon and energy. Based on these facts, Michaels et al., U.S. Patent No. 5,055,397 describes the use of microorganisms as exploration tools to look for new deposits of petroleum and other useful hydrocarbons.

Various hydrocarbons and petroleum products can also cause problems when they contaminate soil or water, e.g., from leaking underground storage tanks or underwater pipelines. This contamination significantly affects the structure and function of the surrounding microbial communities. Therefore, microorganisms can also serve as indicators for such contamination.

Although microorganisms have been used as indirect indicators for hydrocarbons, standard culture and assay methods can require several days of incubation time, and require special facilities for media preparation and the disposal of cultures. For example, one method for detecting microorganisms resistant to hydrocarbons requires analyzing the genes of such microorganisms that are involved in degrading crude oil (see, e.g., U.S. Patent No. 6,265,162).

### SUMMARY

The present invention relates to the intelligent design of devices (such as microarrays) and the methods of making and using such devices to analyze samples, such as environmental samples (e.g., soil, sand, clay, water, and air), to detect the presence of hydrocarbons such as petroleum and polycyclic aromatic hydrocarbons (PAHs). In one aspect, the devices include various probes used to perform parallel screening of a number of analytes that may be present in the samples. The probes are clustered on the devices based on analytes for targets, such as microorganisms, associated with the presence of hydrocarbons or petroleum products. In another aspect, the devices are used to perform parallel screening of a number of hydrocarbon-associated analytes. The new methods and devices can be used to detect hydrocarbon deposits, such as petroleum deposits, and to detect hydrocarbon contaminants in soil and water samples.

In general, the invention features a method of detecting a hydrocarbon (e.g., petroleum) in a sample (e.g., a soil, sand, or water sample) by (a) obtaining a sample; (b) obtaining an array of probes on a substrate, wherein the probes specifically bind to a plurality of analytes (e.g., nucleic acids, antigens, or enzymes) of one or more targets associated with the hydrocarbon (e.g., microorganisms such as bacteria or fungi), and wherein there are at least two different types of analytes for each target; (c) contacting the sample to the probes in the array under conditions that enable the probes to specifically bind to any analytes in the sample to form complexes; and (d) detecting complexes on the array; wherein the presence of a complex indicates the presence of the hydrocarbon in the sample.

In the new methods, there can be at least two targets associated with the hydrocarbon, and the methods can be used to detect multiple hydrocarbons in the sample. For example, the two different types of analytes for each target can be a nucleic acid molecule, e.g., a ribosomal ribonucleic acid (rRNA) molecule and a surface antigen specific for the target or a gene (e.g., the *pmoA* gene) that encodes an enzyme involved in metabolism of the hydrocarbon of the target.

Various hydrocarbons that can be detected using the new methods are listed in Table II, and include petroleum, PAHs, toluene, benzene, or a chlorinated hydrocarbon. Tables II and IV also list microorganisms that can be targets. In specific embodiments, the hydrocarbon can be a PAH, and the targets can be any one or more of the

microorganisms listed in Table V. In other embodiments, the hydrocarbon can be toluene, and the targets are any one or more of: *Alcaligenes faecalis*, *Pseudomonas aeruginosa*, *Pseudomonas alcaligenes*, *Pseudomonas fluorescens*, *Pseudomonas paucimobilis*, *Pseudomonas pickettii*, *Pseudomonas putida* strains F1, mt2 PaW1 (DSMZ 3931), mt 14-26 (DSMZ 3934), *Pseudomonas stutzeri*, *Ralstonia sp.* strain PHS1, or *Stenotrophomonas maltophilia* strain T3-c. The hydrocarbon can also be jet fuel, and the targets can be any one or more of: *Sphingomonas sp.* strain ANT23 and strain ANT17, or the hydrocarbon can be gasoline, and the targets can be any one or more of: *Pseudomonas aeruginosa*, *P. stutzeri*, *P. alcaligenes*, *P. pickettii*, *P. paucimobilis*, *P. putida*, *P. fluorescens*, *Alcaligenes faecalis*.

In another embodiment, the invention features a method of detecting petroleum in a sample by (a) obtaining a sample (e.g., soil, sand, or water); (b) obtaining an array of probes on a substrate, wherein the probes specifically bind to analytes of at least ten (e.g., at least 20) different microorganisms (e.g., listed in Tables II and/or IV) associated with petroleum, wherein there are at least two different types of analytes for each microorganism, and wherein the microorganisms are selected from Table II or Table IV; (c) contacting the sample to the probes in the array under conditions that enable the probes to specifically bind to any analytes in the sample to form complexes; and (d) detecting complexes on the array, wherein the presence of a complex indicates the presence of petroleum in the sample.

In this method, analytes for each microorganism can be a nucleic acid molecule (e.g., a genomic nucleic acid molecule or a ribosomal ribonucleic acid (rRNA) molecule) and a surface antigen specific for the microorganism. For example, the gene can be one that encodes an enzyme involved in metabolism of a petroleum hydrocarbon, such as the *pmoA* gene. The array of probes can include at least twenty different probes, each probe specifically binding to a different one of the at least two different analytes for each of the at least ten different microorganism. The microorganisms can be any ten (or 20) or more of: *Pseudomonas aeruginosa*, *P. stutzeri*, *P. alcaligenes*, *P. pickettii*, *P. fluorescens*, *P. putida*, *P. paucimobilis*, *Alcaligenes faecalis*, *Agrobacterium rubi*, *Alcaligenes faecalis*, *Alcaligenes sp.*, *Arthrobacter oxydans*, *Azospirillum sp.*, *Bacillus macroides*, *Bordetella parapertussis*, *Microbacterium lacticum*, *Microbacterium laevaniformans*, *P. agarici*, *P. corrugata*, *P.*

*pseudoalcaligenes*, *P. syringae*, *Ralstonia sp. strain PHS1*, *Rhodococcus marinoascens*, *Sphingomonas yanoikuyae*, *Stenotrophomonas maltophilia*.

In another aspect, the invention features a device for detecting a hydrocarbon that includes (a) a substrate having a surface; and (b) an array of probes bound to the surface, wherein the probes specifically bind to a plurality of analytes of ten or more (e.g., 20 or more) targets (e.g., microorganisms and heavy metals) associated with the hydrocarbon, wherein there are at least two different types of analytes for each target, and wherein the array of probes comprises at least twenty different probes, each probe specifically binding to a different one of the at least two different analytes for each of the at least ten different targets. The microorganism targets can be those in Table II.

In another aspect, the invention features devices that include (a) a substrate having a surface, wherein the surface has an array of protrusions having top surfaces; and (b) an array of probes, wherein the probes specifically bind to proteins and nucleic acid analytes, and wherein the array of probes is attached to the top surfaces of the protrusions. In these devices, the substrate can be a silicon, silicon dioxide, plastic, metal, metal alloy, zeolyte, polymer, or gold.

Other devices include (a) a substrate having a surface, wherein the surface has an array of wells; and (b) an array of probes, wherein the probes specifically bind to proteins and nucleic acid analytes, and wherein the array of probes is attached within the wells. In these devices, the wells can have mixing devices, such as fans, and can further include electrical connections, wherein the electrical connections connect the mixing devices to an energy, e.g., voltage, source. In addition, the mixing devices can be biological molecules powered by biologic reactions, e.g., based on ATPase, kinesin, kinesin related proteins, myosin, DNA Helicase, DNA Sliding clamps, nucleic acid based rotaxanes and Pseudo-rotaxanes, circular triplex forming oligonucleotides (CTFO), duplex DNA; as well as chimeras and derivatives of such proteins and nucleic acids. The protrusions or wells can have mixing device(s) powered by electromagnetic radiation or the piezoelectric effect or other sources of energy.

In addition, the new devices can include (a) a substrate having a surface; (b) an array of probes, wherein the probes specifically bind to protein or nucleic acid analytes; and (c) an array of linkers, wherein the linkers bind the probes to the surface, and wherein the linkers have different lengths. In these devices, the substrate can be planar, and the linkers can be molecules of polyethylene glycol.

A probe is an antibody, antigen, protein, nucleic acid such as RNA or DNA, or other molecule or compound that specifically binds to an analyte from a target. A target is a marker or indicator for the presence of a hydrocarbon, such as petroleum, in a sample. For example, a target can be a microbe, such as a bacterium, virus, fungus, or a plant such as algae, or some other material known to be associated with hydrocarbon deposits, such as heavy metals, like copper, mercury, and chromium, or the hydrocarbons themselves. An analyte is any biological or chemical molecule or material that is on, within, produced by, or associated with, a target microbe or material, such as an enzyme, a surface antigen, a toxin, a chemical, or a nucleic acid (such as DNA or RNA). In some cases, the analyte can be the target itself, e.g., a heavy metal. In each case, the analyte is detected and/or quantitated by a probe, which specifically binds to the analyte.

A molecule that "specifically binds" to an antigen or other analyte is a molecule that binds to the analyte, but does not substantially bind to other molecules in a sample, e.g., a soil sample, which naturally contains the analyte. Thus, the probes of the invention, such as antibodies and nucleic acid molecules, specifically bind to analytes, such as antigens and DNA or RNA.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

The invention provides several advantages. All relevant tests for a given hydrocarbon, such as petroleum, can be rapidly analyzed with one device, under the same reaction conditions, thereby reducing test costs, while providing a comprehensive, standardized result in a rapid fashion. In addition, the new methods and devices provide facile and low cost alterations and augmentations of the devices to include additional tests, which is cheaper than the laborious and costly process of adding new

tests to a battery of tests conducted separately, each using a separate sample, e.g., from a large tract of land.

Another advantage is that the invention provides methods for detecting almost any biological analyte, such as nucleic acids as well as non-nucleic acid components, in a mixture simultaneously. The new devices can also be used for parallel processing of a large number of (same or different) samples, providing a high-throughput environment. For example, multiple sets of microarrays can be deposited onto a single biochip, which enables screening of multiple samples.

The new systems also provide easy and simple read-out of results by simple visual inspection, and in some embodiments simplify sample handling by combining sample collection and analysis modules to circumvent the need to transfer samples from collection tubes to detection devices. The new methods and devices can also provide better and newer sample mixing during an assay, which improves the quality as well as reduces the time needed to perform an assay.

Another advantage is that the microarray-based detection methods can be easily automated and the new devices can be used with the robots and technologies currently used in many testing laboratories. This will cut down on the costs for incorporating this new technology into an existing laboratory environment. In addition, another advantage to the microarray-based methods is that they can be incorporated into portable devices.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

### DESCRIPTION OF DRAWINGS

FIGS. 1A to 1C are schematic drawings of three biochip devices for detecting petroleum products and hydrocarbons, each having a different configuration of probes in "arrays of arrays" format. For example, FIG. 1A illustrates probes in an "X" configuration, FIG. 1B shows probes in a "V" configuration, and FIG. 1C shows probes in a "+" configuration.

FIGS. 2A to 2C are schematic drawings of detection devices using a 16-microwell format. FIG. 2A is a top view of an enlarged microwell and a solid support, FIG. 2B is a cross-sectional view, and FIG. 2C is a view of one micro-well in three dimensions.



FIGs. 3A and 3B are schematic drawings of a new probe attachment technology using molecular linkers, such as polyethylene glycol (PEG), to attach the probes to a solid support. This new attachment technology can be used in conjunction with the new biochip detection devices.

FIG 4A is a schematic drawing of a mixing system incorporated into a multiwell biochip device.

FIG 4B is an enlarged view of a single well in the multiwell biochip of FIG 4A.

FIGs. 5A to 5C are a schematic drawings of a new inverted array system of multiwell device, that can be used with biochip devices. The substrate includes raised or elevated structures, such as cylinders, onto which specific probe arrays, or arrays of arrays, can be deposited. FIG 5A shows an enlarged well of the multiwell device shown in FIG 5B. FIG 5B shows a cross-sectional view, and FIG 5C shows three-dimensional views of the wells in the multiwell device.

FIGs. 6A to 6E are a series of schematic diagrams of an alternative inverted array system (top view, FIG. 6A, cross-sectional view, FIG. 6B) as used with a microtiter plate (top view FIG. 6C, cross-sectional view, FIG. 11D). FIG 6E shows the inverted array inserted into the microtiter plate.

FIGs. 7A to 7C are schematic representations of a three-dimensional porous array. Such a three-dimensional porous arrays can be manufactured in a number of ways and these figures illustrate one methodology in which the three-dimensional solid-substrate is an array filled with holes. The holes are filled with a gel-like matrix or other materials such as nitrocellulose membranes. The probes can either be pre-bound to the matrix or can be placed subsequent to matrix deposition step.

FIGs. 8A to 8C are diagrams of another three-dimensional porous array. Such a three-dimensional porous array can be manufactured in a number of ways and these figures illustrate one method.

FIG. 9 is a schematic drawing of a microfluidic concentrator biochip.

FIGs. 10A to 10E are a series of representations showing how strand-invader molecules can achieve hybridization enhancement.

FIGs. 11A to 11G are a series of schematic drawings of novel hybridization chambers and their various parts.

FIG. 12 is a representation of the principal behind the new UniScreen™ Technology, which allows detection of any analyte, such as DNA, RNA, and proteins, in a single multiplexed assay.

### DETAILED DESCRIPTION

5        There is a need for a comprehensive method to detect hydrocarbons, such as those found in petroleum, in environmental samples. In the new detection devices or kits, multiple tests are run in parallel, to avoid delays in detection due to iterative and sequentially performed individual tests. The new devices can immensely simplify the process of prospecting for hydrocarbons and petroleum products, e.g., for prospecting  
10    for new sources of these materials, or for assaying soil or water contaminated with these materials.

#### General Methodology

      The present invention provides methods for intelligently combining many tests  
15    into one test kit or device. The methods enable the clustering or multiplexing of tests (e.g., probes) specific to various microorganisms that associate with specific hydrocarbons to provide devices and assays for performing multiple tests in parallel for one or more specific hydrocarbons and/or petroleum products. The new methods enable facile and low cost alterations and augmentations of the devices to include  
20    additional tests, which is cheaper than the laborious and costly process of adding new tests to a battery of tests conducted separately, each using a separate sample. The test kits are user-friendly and use a simple format to provide rapid identification of samples that indicate the presence of either a specific hydrocarbon, or a group of multiple hydrocarbons. These results are relevant in the detection of new deposits of  
25    hydrocarbons such as petroleum, as well as for locating sites contaminated by hydrocarbons, such as jet fuel, gasoline, or polycyclic aromatic hydrocarbons.

      Oil exploration and prospecting, as well as the detection of hydrocarbon-contaminated sites, requires sensitive surface analysis methods to detect low molecular weight hydrocarbon seepages and concentrations (see, e.g., Wagner et al., MicroPro  
30    GmbH.; Microbial Prospection for Oil and Gas Onshore and Offshore in North-West Europe. 2000; see also Internet sites such as micropro.de and e-b-t.com)(see also, Murrell et al., Archives of Microbiology, 173, 325-332, 2000; Cheng YS et al., Applied

and Environmental Microbiology, 65(2), 648-651, 1999; Gilbert B et al., Applied and Environmental Microbiology, 66(3), 966-975, 2000; Wise MG et al., Applied and Environmental Microbiology, 65(11), 4887-4897, 1999; and Microbial Oil Prospecting, U.S. Patent No. 5,093,236).

- 5        The increased hydrocarbon supply, in the form of liquids or gases, above oil fields or waste sites creates ideal conditions for development and growth of select species of microorganisms, fungi, and plants. For example, polycyclic aromatic hydrocarbon (PAH) degrading bacteria have adapted metabolisms that oxidize low gaseous hydrocarbons. Table I lists a number of groups of bacteria, and certain species,  
10    that metabolize hydrocarbons.

Table I

Hydrocarbon Degrading Microorganisms		
	Group	Species
15	Pseudomonas	<i>Pseudomonas fluorescens</i>
	Achromobacter	<i>Achromobacter xylosoxidans</i>
	Arthobacter	
	Micrococcus	
	Nocardia	<i>Nocardioles Luteus</i>
	Bibrio	
20	Acinetobacter	
	Bacillus	
	Brevibacterium	
	Corynbacterium	
	Flavobacterium	<i>Flavobacterium gondwanese</i>
25	Halomonas	
	Alealigenes	
	Sphingomones	
	Mycobacterium	
	Rhodococcus	
	Cycloclasticus	
30	Vibrio	<i>Vibrio Aguillarum</i>

Table II recites various specific hydrocarbons and a partial list of bacteria and fungi that can be used to indicate the presence of these hydrocarbons. The bacteria and

fungi in Table II can be used as targets in the new methods by selecting as analytes nucleic acids, protein antigens, enzymes, or other molecules that are unique to these bacteria. Each new biochip or kit will include probes that specifically bind to two or more different analytes for each target, e.g., microorganism.

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**Table II. Specific Hydrocarbons and Their Associated Microorganisms**

10	<b>Ethylbenzene:</b> <i>Alcaligenes faecalis</i> , <i>Azoarcus</i> sp. strain EbN1, <i>Pseudomonas alcaligenes</i> , <i>Pseudomonas fluorescens</i> , <i>Pseudomonas pickettii</i> , <i>Pseudomonas stutzeri</i> , <i>Pseudomonas paucimobilis</i> , <i>Ralstonia</i> sp. strain PHS1, <i>Stenotrophomonas maltophilia</i> T3-c.
15	<b>Benzene:</b> <i>Agrobacterium rubi</i> , <i>Alcaligenes faecalis</i> , <i>Alcaligenes</i> sp., <i>Arthrobacter oxydans</i> , <i>Azospirillum</i> sp., <i>Bacillus macroides</i> , <i>Bordetella parapertussis</i> , <i>Microbacterium lacticum</i> , <i>Microbacterium laevaniformans</i> , <i>Pseudomonas aeruginosa</i> , <i>Pseudomonas agarici</i> , <i>Pseudomonas alcaligenes</i> , <i>Pseudomonas corrugata</i> , <i>Pseudomonas flavescens</i> , <i>Pseudomonas paucimobilis</i> , <i>Pseudomonas pickettii</i> , <i>Pseudomonas pseudoalcaligenes</i> , <i>Pseudomonas putida</i> , strain PB4, <i>Pseudomonas stutzeri</i> , <i>Pseudomonas syringae</i> , <i>Ralstonia</i> sp. strain PHS1, <i>Rhodococcus marinoascens</i> , <i>Sphingomonas yanoikuyae</i> , <i>Stenotrophomonas maltophilia</i>
20	<b>Toluene:</b> <i>Alcaligenes faecalis</i> , <i>Pseudomonas aeruginosa</i> , <i>Pseudomonas alcaligenes</i> , <i>Pseudomonas fluorescens</i> , <i>Pseudomonas paucimobilis</i> , <i>Pseudomonas pickettii</i> , <i>Pseudomonas putida</i> strains F1, mt2 PaW1 (DSMZ 3931) and mt 14-26 (DSMZ 3934), <i>Pseudomonas stutzeri</i> , <i>Ralstonia</i> sp. strain PHS1, <i>Stenotrophomonas maltophilia</i> strain T3-c
25	<b>Styrene:</b> <i>Alcaligenes</i> sp., <i>Azospirillum</i> sp., <i>Microbacterium laevaniformans</i> , <i>Pseudomonas aureofaciens</i> , <i>Pseudomonas corrugata</i> , <i>Pseudomonas syringae</i> , <i>Rhodococcus globerulus</i> , <i>Rhodococcus marinoascens</i>
30	<b>Xylenes:</b> <i>Alcaligenes faecalis</i> , <i>Pseudomonas aeruginosa</i> , <i>Pseudomonas alcaligenes</i> , <i>Pseudomonas pickettii</i> , <i>Pseudomonas putida</i> strain mt-2, <i>Pseudomonas stutzeri</i> , <i>Ralstonia</i> sp. strain PHS1, <i>Stenotrophomonas maltophilia</i> strain T3-c
35	<b>TMB (1,2,4-trimethylbenzene):</b> <i>Alcaligenes faecalis</i> , <i>Pseudomonas aeruginosa</i> , <i>Pseudomonas alcaligenes</i> , <i>Pseudomonas pickettii</i> , <i>Pseudomonas putida</i> , <i>Pseudomonas stutzeri</i>
40	<b>Biphenyl:</b> <i>Alcaligenes</i> sp. strain ATCC53640; <i>Pseudomonas pseudoalcaligenes</i> , strain KF707, <i>Pseudomonas putida</i> ATCC53643, <i>Pseudomonas</i> sp. strain ATCC53643, <i>Pseudomonas</i> sp. strain LB400b, <i>Sphingomonas paucimobilis</i> strain DSMZ 6900, <i>Sphingomonas Yanoikuyae</i> strain Q1
40	<b>Jet Fuel (JP8):</b> <i>Sphingomonas</i> sp. strain ANT23, strain ANT17
45	<b>Regular-Grade Unleaded Gasoline:</b> <i>Pseudomonas aeruginosa</i> , <i>P. stutzeri</i> , <i>P. alcaligenes</i> , <i>P. pickettii</i> , <i>P. paucimobilis</i> , <i>P. putida</i> , <i>P. fluorescens</i> , <i>Alcaligenes faecalis</i>
45	<b>Hexane:</b> <i>P. stutzeri</i> , <i>P. alcaligenes</i> , <i>P. pickettii</i> , <i>P. paucimobilis</i> , <i>P. putida</i> , <i>Alcaligenes faecalis</i>
50	<b>Octane:</b> <i>Pseudomonas aeruginosa</i> , <i>P. stutzeri</i> , <i>P. alcaligenes</i> , <i>P. pickettii</i> , <i>P. fluorescens</i> , <i>Alcaligenes faecalis</i>
50	<b>Decane:</b> <i>Pseudomonas aeruginosa</i> , <i>P. stutzeri</i>

**Cyclohexane:** *Pseudomonas aeruginosa*, *P. stutzeri*, *P. alcaligenes*, *P. pickettii*, *P. paucimobilis*, *P. fluorescens*, *Alcaligenes faecalis*

5 **Cycloheptane:** *Pseudomonas aeruginosa*, *P. stutzeri*, *P. alcaligenes*, *P. pickettii*, *P. paucimobilis*, *P. putida*, *P. fluorescens*, *Alcaligenes faecalis*

**Methylcyclopentane:** *Pseudomonas aeruginosa*, *P. stutzeri*, *P. alcaligenes*, *P. pickettii*, *Alcaligenes faecalis*

10 **TMP (2,2,4-trimethylpentane):** *Pseudomonas aeruginosa*, *P. stutzeri*, *P. alcaligenes*, *P. pickettii*, *Alcaligenes faecalis*

**O-Xylene:** *Pseudomonas aeruginosa*, *P. stutzeri*, *P. alcaligenes*, *Alcaligenes faecalis*

15 **P-Xylene:** *Pseudomonas aeruginosa*, *P. stutzeri*, *P. alcaligenes*, *P. pickettii*, *P. putida*, *Alcaligenes faecalis*

### **Chlorinated Hydrocarbons**

20 **Dichloroethane:** *Ancylobacter aquaticus* strains AD20, AD25, and AD27, *Pseudomonas sp.* strain DCA1, *Xanthobacter autotrophicus* strain GJ10

**Vinyl Chloride:** *Pseudomonas aeruginosa*, strain MF1

25 **Polyvinyl Chloride:** *Phanerochaete chrysosporium* (white rot fungi)

**Polycyclic Aromatic Hydrocarbons (PAHs):** *Sphingomonas aromaticivorans* F199, *Sphingomonas yanoikuyae* B1

30 **Phenanthrene:** *Mycobacterium sp.* strain 'FSPHF', *Pseudomonas putida* strains PB4 'Dw 2-21', *Sphingomonas paucimobilis* strains (DSMZ 6900), TB4 and 'PF I', *Sphingomonas sp.* strains RP003, RP006, WP01, *Xanthomonas maltophilia* strain 'MK Phe'

35 **Anthracene:** *Mycobacterium sp.* stain 'FFSPH', *Pseudomonas sp.* Strain (NCIMB 12229), *Sphingomonas paucimobilis* strain 'Ba II', *Xanthomonas maltophilia* strains 'MK Anth I', and 'MK Anth II'

**Pyrene:** *Gordona sp.* strain 'bp9', *Mycobacterium gilvum* strain (DSMZ 9487), *Rhodococcus sp.* strain 'UW I'

40 **Fluoranthene:** *Mycobacterium sp.* strain 'FFSPH', *Sphingomonas paucimobilis* strains 'PF I', EPA505

45 **Naphthalene:** *Alcaligenes faecalis*, *Pseudomonas aeruginosa*, *Pseudomonas alcaligenes*, *Pseudomonas fluorescens* strains (DSMZ 6505), *Pseudomonas putida* strains PaW 736 (DSMZ 4302/NCIB 9816), PpG7 (DSMZ 4476), (DSMZ 50222), (DSMZ 50208), and ATCC17484, *Pseudomonas stutzeri*, *Pseudomonas. Pickettii*, *Pseudomonas sp* strains 'MK Naph I', 'MK Naph II', 'MK Naph III', ATCC17483, *Sphingomonas paucimobilis* strain (DSMZ 6900), *Xanthomonas maltophilia* strains 'MK Phe', 'MK Anth I', and 'MK Anth II'

Similarly, methanotrophic bacteria have adapted metabolisms that oxidize methane as their sole source of carbon and energy (see, e.g., Wrenn B, Venosa A; Selective enumeration of aromatic and aliphatic hydrocarbon degrading bacteria by a

most probable number procedure, *Canadian Journal of Microbiology*, 42 (3), 242-248, 1996). Table III lists methanotrophic bacteria.

**Table III. Methanotrophs**

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Type	Group	Species
1	Methylomonas	<i>Methylomonas Methanica</i>
1	Methylomicrobium	<i>Methylomicrobium Album</i>
1	Methylobacter	<i>Methylobacter Luteus</i>
1	Methylocaldum	<i>Methylocaldum tepidum</i>
1		<i>Methylocaldum szegediense</i>
1		<i>Methylocaldum Gracile</i>
1	Methylosphaera	<i>Methylosphaera hansonii</i>
X	Methylococcus	<i>Methylococcus Capsulatus</i>
X		<i>Methylococcus Luteus</i>
2	Methylocystis	<i>Methylocystis Parvus</i>
2	Methylosinus	<i>Methylosinus Trichosporium</i>

All of these microorganisms live in soil above oil and gas fields or other hydrocarbon deposits in the deeper subsurface and represent a cost-effective method for preliminary exploration in unknown areas and an accurate basis for ranking seismically detected geological structures. In addition, there are numerous hydrocarbon metabolizing microorganisms other than bacteria, such as fungi (e.g., *Phanerochaete chrysosporium*, *Cladosporium sphaerospermum*, *Emericella nidulans*, *Graphium putredinis*, *Eupenicillium javanicum* and *Aspergillus flavipes*.; also see, e.g., Hammel, Environ Health Perspect, 103(Suppl 5) 1995; and Oudot et al., Soil Biol Biochem, 25:1167-1173, 1993), and plants (see, e.g., PhytoPet©, A database of Plants That Play a Role in the Phytoremediation of Petroleum Hydrocarbons, Farrell et al., Proc. Second Phytoremed. Tech. Sem., Environment Canada, Ottawa, ON, pp. 29-40, 2000), which have been found to live on the soil-surface above oil fields.

The invention features a biochip based test kit for detecting the presence or absence of such microorganisms in soil samples from potential oil exploration and bioremediation sites. Since the metabolism of these microorganisms has adapted to utilize hydrocarbons as a sole source of energy, specific genes like pmoA, which

encode adapted metabolic enzymes, can serve as analytes that are accurate markers for the presence of hydrocarbons in a sample.

For example, the process of methane oxidation is initiated by the methane monooxygenase enzyme. All known methanotrophs produce a membrane-associated particulate methane monooxygenase (pMMO) and some produce a cytoplasmic soluble methane monooxygenase (sMMO), which is expressed only under copper limiting conditions. The pMMO enzyme is encoded by three genes in the pmoCAB operon. The pmoA gene has been shown to be highly conserved among methanotrophs and can be used to detect these organisms in the methane rich environments that exist above oil and gas fields. Furthermore, two types of methanotrophs can be distinguished on the basis of their biochemical differences.

As shown in Table III, Type 1 methanotrophs are  $\gamma$ -proteobacteria divided into the 6 subgroups: Methylomonas, Methylobacter, Methylochromium, Methylococcus, Methylocaldum, and Methylosphaera. They oxidize methane to produce methanol and then assimilate formaldehyde via the ribulose monophosphate cycle. Type 2 methanotrophs are  $\alpha$ -proteobacteria of subgroups Methylosinus and Methylocystis and they further assimilate formaldehyde into carbon via the serine cycle. These differences can be analyzed using a biochip to quantitate different sub-species of hydrocarbon oxidizing bacteria.

Since these microorganisms have adapted to utilize hydrocarbons as a sole source of energy, specific genes for adapted metabolic enzymes, such as methyl monooxygenase (which oxidizes methane gas), can serve as accurate markers in an assay. These markers can be used as targets for probes on the biochip platform to perform a comprehensive analysis of soil and other samples to determine the presence of specific microorganisms as an indicator of the presence of hydrocarbon oils or gas under the surface. As noted above, microorganisms that can be used for this purpose are presented in Tables I, II, and III. Additional microorganisms, materials, and plants, can be used as targets. For example, the microbes can be methylotrophs as well as other small-chain hydrocarbon oxidizing species.

The new biochip-based products will be highly accurate because they identify microorganisms not by whether they can grow on a certain culture medium or how long it takes them to grow, but whether they have certain genes or proteins, e.g., the genes or proteins that allow the organism to utilize hydrocarbons as an energy source. This

technique is much faster than the current methods of detection, and is far more comprehensive, because a number of different microorganisms can be detected in a single multiplexed assay.

In addition, by selecting multiple surface antigens, enzymes, nucleic acids, and other molecules that are unique to these microorganisms as additional analytes, the new methods and devices can include multiple probes for the same microorganism, which provides much more accurate results and avoids false positives. Thus, the new methods and devices provide excellent sensitivity as well as the selectivity. Furthermore, the new methods and devices can be tailored to detect specific hydrocarbons, groups of several hydrocarbons, or the class of all hydrocarbons. Thus, the new methods and devices can be as specific or general as desired.

The format of the new multiplexed devices provides a new approach to prospecting for hydrocarbons such as petroleum. The new devices detect analytes at the molecular level within a sample (such as, e.g., water, soil, sand, or air (e.g., looking for gases such as methane) using an array having a plurality of probes to which the sample is applied. In one embodiment, the detecting device can employ microarray technology to cluster many probes onto a single biochip. However, the new devices and methods are not limited to biochips or microarrays. Other technologies can be used to create such devices. For example, a multiplexed device can be based on bead array technologies or microfluidic array technologies (from companies such as Luminex, Illumina, Aclara, and Caliper). As described herein, there are a number of ways of making multiplexed arrays.

The new methods enable the use of multiple probes that are all bound to a substrate using methods that keep the mobilized probe molecules biologically active. The multiplexed devices also enable the simultaneous use of numerous disparate tests/probes under the same reaction conditions with high sensitivity and specificity.

Another feature of the new methods and devices is that numerous types of analytes, including nucleic acids and non-nucleic acid analytes such as antigens on the surface of microorganisms can be simultaneously detected and/or quantitated on the same device. A number of naturally occurring as well as synthetic molecules recognize nucleic acids under physiological conditions and can be used as probes. Examples of such probe molecules include transcription factors, such as zinc-finger proteins (ZFPs), Helix-Turn-Helix motif proteins (e.g., GATA-1), immunoglobulin motif proteins (e.g.,



NFkB, NFAT), and polyamides, such as oligomeric heterocyclic minor groove binders (MGBs). Advantages of using polyamide molecules (such as ZFPs and MGBs) include: 1) they bind to double-stranded nucleic acids, and 2) they do so under almost the same conditions as proteins use to bind to other proteins and other molecules. The idea is to select a target nucleic acid as an analyte, e.g., a gene known to encode an enzyme involved in hydrocarbon metabolism, or a ribosomal RNA that is unique to a specific genus or species of microorganism, and then to design a polypeptide-based probe, such as a ZFP or MGB that specifically binds to the nucleic acid analyte.

ZFPs are transcription factors in eukaryotes (e.g., in yeasts, plants, and mammals), which contain the Cis2His2 class of zinc finger domains, identified first in the DNA and RNA binding transcription factor TFIIIA, as their DNA-binding modules. This class of zinc finger motifs is unique in that their DNA binding specificities are highly adaptable; unlike most other DNA-binding domains, dozens of zinc finger domains characterized thus far bind to highly diverse DNA sequences, with each zinc finger domain able to recognize distinctive DNA binding sites.

DNA binding zinc fingers related to those of the mouse transcription factor Zif268 have been used successfully as scaffolds in the design of DNA binding proteins with predetermined sequence specificity. The small size of the zinc finger limits individual modules to the recognition of only a few adjacent base pairs in duplex DNA, but allows multiple tandem modules to wind around the major groove of DNA, thus recognizing a longer run of bases. In the crystal structure of the Zif268 fingers bound to DNA, three modules occupy the major groove of the DNA in series, each making base-specific contacts and overlap typically three to four basepair subsites. Specificity arises from 1:1 interactions between residues of each zinc finger [alpha]-helix and the corresponding DNA bases. Zinc fingers have also been used to bind to DNA-RNA hybrids, RNA duplexes, and nucleic acids containing modified bases.

Simple covalent tandem repeats of the zinc finger domain allow for the recognition of longer asymmetric sequences of nucleic acids. Such adaptability of zinc finger domains in DNA/RNA recognition can be used to isolate or design novel proteins with altered DNA/RNA binding specificities, and to construct tailor-made nucleic acid binding proteins that specifically recognize almost any predetermined DNA/RNA sequence. For example, phage display technology can be used to create novel zinc finger proteins that bind diverse sequences with high affinity and specificity.

Such novel or "designer" zinc finger proteins with desired nucleic acid binding specificities can serve as efficient probes for detecting nucleic acid sequences in a sample. These proteins are designed based on the DNA and RNA sequences of the microorganisms known to be associated with hydrocarbons, such as the

5 microorganisms described herein.

For additional information on zinc-finger proteins and their use in arrays, see, e.g., Hanas, WO 99/45388 (published on Sept. 10, 1999); and Wagner et al., WO 00/04382 (published on Jan. 27, 2000).

Similarly, MGB polyamides are a class of small synthetic molecules that bind in

10 a sequence-specific manner in the minor groove of double-stranded DNA with extraordinary affinity and specificity. MGBs use a chemical recognition code that can distinguish each of the four Watson-Crick base pairs in the minor groove of DNA. Chemists have applied this binding code to design and synthesize a number of different such molecules that specifically recognize a given target sequence in the human

15 genome. MGBs also bind their target nucleic acids under physiological conditions. Such novel or "designer" minor groove binding ligands with desired nucleic acid binding specificities can also be used as efficient probes for detecting nucleic acid sequences in a sample. Again, using known techniques, these MGBs can be tailored to specifically bind to dsDNA of microorganism, known to associate with hydrocarbons.

20 Transcription factors, such as ZFPs and small molecule polyamide ligands that recognize nucleic acids, such as MGBs, constitute a novel class of probes that recognize and detect nucleic acids under physiological conditions. Conditions used for binding such agents to their target nucleic acid sequences are similar to the ones used for detecting proteins and other non-nucleic acid components. Thus, these agents can

25 be combined with protein and other biologic detecting agents onto a single support, such as a chip, or in a single assay, for a unified screening device (e.g., UniScreen™). Such a device can detect DNA, RNA, proteins, glycoproteins, polysaccharides, and other antigens simultaneously, on a single device (such as a biochip), and under the same conditions (See FIG. 12).

30 The specific biochemical environments required for binding of probe molecules currently limit multi-analyte biochip assays. For instance, proteins necessitate a stable pH and temperature to remain folded and retain optimal binding affinity for the target molecule. Conversely, DNA, PNA, and RNA require thermal cycling for hybridization

of complementary strands to occur. This temperature variation would lower the binding capability of proteins and in most cases completely denature them. Therefore, the possibility of protein and nucleic acid probes binding analyte molecules in the same assay has been difficult before the development of the new methods described herein.

5        However, there are a number of naturally occurring and synthetic molecules that recognize nucleic acids by processes such as strand invasion. Strand invasion involves a modified nucleic acid sequence that hybridizes with duplex DNA and is capable of removing a length of nucleic acid via free energy advantage. Single-stranded DNA, RNA, and peptide nucleic acid (PNA) molecules accomplish strand invasion under  
10        specific conditions. Chimeras of such molecules also result in a strand-invaded complex. In addition, Epoch Biosciences has created a class of synthetic probes called selective binding complementary oligonucleotides (SBCs) that consist of modified nucleotide bases that form hybrids with target duplex nucleic acids. A feature of SBCs is that the two strands do not form a stable duplex with each other, yet they form a very  
15        stable complex with the two strands of a target DNA. These molecules are usually used together, that is, the two complementary sequences are used to perform strand-invasion of DNA.

      Hybridization of DNA or RNA targets to DNA/RNA probes attached to a biochip requires that the target be in single-stranded form. One technique that can be  
20        used in the new methods involves the use of a strand-invader, such as PNA, circular DNA/RNA, or one of two SBCs (an illustration is shown in FIG. 10B), to separate a target nucleic acid analyte duplex into a complex with a single-stranded region. As shown in FIGs. 10A to 10E, this single-stranded target can easily bind to probes bound to a biochip without requiring a thermal denaturation step on the chip. A second, much  
25        smaller, oligonucleotide can also be used in the mixture to drive the reaction to completion. This method is relevant to biochip assays because a DNA probe missing an oligo-size portion of the duplex has extremely high binding affinity for any complementary nucleic acid sequence even under physiological condition (see, e.g., SBC Oligos, Epoch Biosciences; U.S. Patent No. 5,912,340); and Zhang et al., Nucleic  
30        Acids Research, 28, 3332-38, 2000).

      Although the new multiplexed devices and kits have not been previously described, techniques for attaching individual probes to solid substrates are described in various publications such as, for example, U.S. Patent No. 6,110,426; U.S. 5,763,158;

U.S. 6,171,797; WO 00540046; U.S. 5,858,804; U.S. 5,252,743; U.S. 5,981,180; U.S. 6,083,763; WO 0004390; WO 00104389; WO 00104382; and other related publications.

5       The new devices/systems include many useful and advantageous features. For example, they can detect analytes under uniform temperature and pressure conditions, and can also have reactive sites/arrays that are either open or are enclosed in a chamber. These chambers can be flow-through or non flow-through. The devices/systems can also be entirely sealed once a sample has been introduced. In other embodiments, the new systems/devices combine sample collection modules with detection devices into a  
10   unique combined module, which circumvents the need to transfer samples from collection tubes etc. to the detection devices. Alternatively, the systems/devices include sample collection modules that are separate from detection device modules and that can easily be connected at any stage, without having to take the sample out of the collection module.

15       The new multiplexed detection systems enable a number of disparate tests to be performed simultaneously and under the same conditions, with low cross-reactivity and with high sensitivity and specificity. The devices/systems also provide a better dynamic range than currently available systems, and provide simple data interpretation as well as accurate detection information.

20       The new devices/systems also reduce the amount of sample needed. Individual tests as currently performed each require a certain amount of sample. As the number of tests goes up, the required amount of sample also goes up. The new multiplexed assay devices overcome such limitations in testing and sample requirements, because the sample size stays generally the same, even when new test probes are added to the  
25   system.

      The new devices/systems also can be used with an "array of arrays" format, which provides a single device that can be used to process a large number of (same or different) samples in parallel, thus providing a high-throughput environment. For example, by introducing multiple sets of microarrays on a single biochip, one can  
30   screen multiple samples with clustered tests in one step.

      The new devices can be processed and analyzed using standard automated processing, such as robotic and computer-controlled screening, scanning, and delivery of results. The new devices, such as detection biochips, can be processed using

multiple dyes/colors. Assays can be performed either simultaneously or sequentially. Assays can use either single unique probes for each analyte or multiple unique probes for each analyte. Different types of assays, such as sandwich immunoassays, competition immunoassays, catalytic antibodies, hybridization, and single base extension, can be used in the new methods.

The new methods and systems also provide for image storage and processing, and manipulating stored signals to form a new image, all using standard techniques. Such methods can provide test results in formats that are easy to read and interpret. The new methods also include placing optical positioning markers for automated image processing and read-out. For example, such a method can include a row of dilution series for dynamic range determination and internal calibration on each biochip/microarray. The new methods generally provide excellent assay results by improving as well as optimizing currently used sample mixing conditions during the assay.

In some embodiments, the new methods include methods of delivering the results via communications networks such as the Internet, telephone systems, or wireless communication systems.

#### **Methods of Preparing Hydrocarbon Detection Devices**

The detection devices are based on a variety of substrate-based technologies, such as solid plates, chips, or slides, as well as solid beads or microparticles. For example, the new devices can use microarray technology (see FIGs. 1A to 1C as an example). Glass or silicon microscope slides/chips can be used to prepare the devices (FIG. 1A). Alternatively, a larger membrane can be used to prepare up to 96 wells/sites per slide. A number of other materials, such as plastics, polymers, metals, and metal alloys can also be used as substrates. The device can have one or more sites per slides. The substrates, e.g., glass slides, can be coated with an organic or inorganic material to improve the surface properties as well as covalently attach the probes. If membranes are used as the substrate for the probes, they should not require pretreatment, but can be pretreated. A number of different targets are detected in a single assay by using one or more arrays of immobilized capture probes on the substrate (e.g., slide) surface. Methods of selecting and clustering the probes are described below.

### Selecting Targets and Analytes That are Associated with Specific Hydrocarbons

For any given hydrocarbon, there can be one, two, dozens, or possibly hundreds of targets associated with the hydrocarbon. Each target will have at least one analyte, and typically two or more analytes, e.g., a surface antigen or a specific protein, such as an enzyme, that are unique to the target (or class or group of targets), e.g., one or more microbes such as bacteria, viruses, mycoplasma, protozoa, plant cells (such as algae and pollens), or fungi. An analyte can also be a nucleic acid, such as a gene or ribosomal RNA, that is unique to a particular microorganism or group of microorganisms, e.g., related microorganisms. The regions or segments of nucleic acids, e.g., DNA or RNA, that are specific for a particular target microbe can be found, e.g., in GenBank, or in other various publicly available databases.

For example, one can often find ribosomal RNA (rRNA), or DNA that encodes such rRNA, that is unique not only to a genus of bacteria, but to a particular species as well. These unique regions of rRNA have been published for numerous microbes. For target microbes for which such unique rRNAs have not yet been described, one of skill can locate such unique sequences and prepare probes that hybridize specifically using standard techniques. For discussions of how to locate such unique nucleic acid sequences and to use them as targets for specific nucleic acid probes, see, e.g., Hogan et al., U.S. Patent Nos. 5,595,874 and 6,150,517.

Surface proteins and other antigens of microorganisms, fungi, or plants, also serve as useful analytes. Antibodies to microbial surface antigens can be generated using standard techniques and can be used as probes in the new methods and devices. For example, surface proteins unique to specific hydrocarbon metabolizing microorganisms can be used as immunogens to generate antibodies, which signal the presence of the microorganism in a sample, using standard techniques for polyclonal and monoclonal antibody preparation. Alternatively, the microorganisms themselves can be used as antigens, or if pathogenic to mammals, the microorganisms can be killed before they are used as antigens.

Such antigens are typically used to prepare antibodies by immunizing a suitable subject (e.g., rabbit, goat, mouse or other mammal) with the immunogen. The preparation can further include an adjuvant, such as Freund's complete or incomplete adjuvant, or similar immunostimulatory agent. Immunization of a suitable subject with

an immunogenic antigen preparation induces a polyclonal anti-microbial antigen antibody response.

Both polyclonal and monoclonal antibodies that bind to the microbial antigens can be used. The term "monoclonal antibody" or "monoclonal antibody composition," as used herein, refers to a population of antibody molecules that contain only one species of an antigen-binding site capable of immunoreacting with a particular epitope of an analyte. A monoclonal antibody composition thus typically displays a single binding affinity for a particular microorganism or surface antigen with which it immunoreacts.

Antibody molecules can be isolated from the mammal (e.g., from the blood) and further purified by well-known techniques, such as Protein A chromatography to obtain the IgG fraction. At an appropriate time after immunization, e.g., when the antibody titers are highest, antibody-producing cells can be obtained from the subject and used to prepare monoclonal antibodies by standard techniques, such as the hybridoma technique originally described by Kohler and Milstein, *Nature*, 256:495-497 (1975).

The technology for producing hybridomas is well known (*see generally Current Protocols in Immunology* (1994) Coligan et al. (eds.) John Wiley & Sons, Inc., New York, NY). Briefly, an immortal cell line (typically a myeloma) is fused to lymphocytes (typically splenocytes) from a mammal immunized with an immunogen as described above, and the culture supernatants of the resulting hybridoma cells are screened to identify a hybridoma producing a monoclonal antibody that binds to the target analyte (e.g., surface antigen or entire microorganism).

Any of the many well known protocols used for fusing lymphocytes and immortalized cell lines can be applied for the purpose of generating a monoclonal antibody (*see, e.g., Current Protocols in Immunology, supra*; Galfre et al. (1977) *Nature* 266:55052; R.H. Kenneth, in *Monoclonal Antibodies: A New Dimension In Biological Analyses*, Plenum Publishing Corp., New York, New York (1980); and Lerner (1981) *Yale J. Biol. Med.*, 54:387-402. Moreover, the ordinarily skilled worker will appreciate that there are many variations of such methods that would also be useful. Typically, the immortal cell line (e.g., a myeloma cell line) is derived from the same mammalian species as the lymphocytes. For example, murine hybridomas can be made by fusing lymphocytes from a mouse immunized with an immunogenic preparation of the present invention with an immortalized mouse cell line, e.g., a

myeloma cell line that is sensitive to culture medium containing hypoxanthine, aminopterin and thymidine ("HAT medium"). Any of a number of myeloma cell lines can be used as a fusion partner according to standard techniques, e.g., the P3-NS1/1-Ag4-1, P3-x63-Ag8.653 or Sp2/O-Ag14 myeloma lines. These myeloma lines are  
5 available from ATCC. Typically, HAT-sensitive mouse myeloma cells are fused to mouse splenocytes using polyethylene glycol ("PEG"). Hybridoma cells producing a monoclonal antibody of the invention are detected by screening the hybridoma culture supernatants for antibodies, e.g., using a standard ELISA assay.

Alternative to preparing monoclonal antibody-secreting hybridomas, a  
10 monoclonal antibody can be identified and isolated by screening a recombinant combinatorial immunoglobulin library (e.g., an antibody phage display library) with the target surface antigen or microorganism to thereby isolate immunoglobulin library members that bind to that antigen/microorganism. Kits for generating and screening phage display libraries are commercially available (e.g., the Pharmacia *Recombinant*  
15 *Phage Antibody System*, Catalog No. 27-9400-01; and the Stratagene *SurfZAP® Phage Display Kit*, Catalog No. 240612). Additionally, examples of methods and reagents particularly amenable for use in generating and screening antibody display library can be found in, for example, U.S. Patent No. 5,223,409; PCT Publication No. WO 92/18619; PCT Publication No. WO 91/17271; PCT Publication No. WO 92/20791;  
20 PCT Publication No. WO 92/15679; PCT Publication No. WO 93/01288; PCT Publication No. WO 92/01047; PCT Publication No. WO 92/09690; PCT Publication No. WO 90/02809; Fuchs et al. (1991) *Bio/Technology* 9:1370-1372; Hay et al. (1992) *Hum. Antibod. Hybridomas* 3:81-85; Huse et al. (1989) *Science* 246:1275-1281; Griffiths et al. (1993) *EMBO J.* 12:725-734.

25 An analyte can also be a specific gene that is not normally present or expressed, or is not present in multiple copies, or includes a mutation in a normally present gene (e.g., that allows the microorganism to thrive in a hydrocarbon environment). The key is that the analyte should be unique to the individual microorganism or group of microorganisms that are to be detected.

30 One goal of analyte selection is to select a number of analytes (i.e., associated with different hydrocarbons and petroleum products) that provides a high level of reliability that one of the selected analytes is in fact indicating the presence of a hydrocarbon. In other words, the goal is to select analytes that are the most likely to be



associated with a particular hydrocarbon or group of hydrocarbons. For example, if there are 50 possible analytes (for 25 different microorganisms) that can be associated with a particular hydrocarbon (or group of hydrocarbons), but only 10 analytes are known to be associated with 90% of the hydrocarbons in the group (or 90% of the time with a single hydrocarbon), then a detecting device might include probes (e.g., 10 or more probes) to detect only those 10 analytes to provide a sufficient level of reliability. This device would not provide a positive result if the analytes in a sample happens to be one of the analytes in the 10% not detected by the device.

A more sophisticated and/or accurate detection device might include an additional set of probes that are specific for 10 more known analytes that together with the first 10 analytes are known to be associated with 99% of the hydrocarbons (or 99% of the time with a single hydrocarbon) of interest.

Of course, other scenarios are possible, and will vary depending on whether the goal of detection is to find specific, individual hydrocarbons or groups of different hydrocarbons, or merely the presence of any hydrocarbons.

To provide a high degree of accuracy, several probes can be used to detect and/or quantitate a specific analyte or specific microorganism (or other material or composition associated with a hydrocarbon deposit). For example, one probe can be designed to specifically bind to one epitope of an antigen analyte, and a second probe can be designed to specifically bind to a second epitope of the same antigen. In another example, one probe can be specific for an enzyme that is produced by a specific microbe, a second probe can be specific for a specific nucleic acid associated with that microbe, and a third probe can be specific for a surface antigen from that microbe. In addition, numerous probes of the same type can be clustered into separate locations or spots on a substrate to ensure that the sample is evenly distributed over the entire array and that even low concentrations of analyte are detected. Two or more probes that recognize different epitopes of an antibody or antigen can also be mixed and placed on the same spot.

There are numerous analytes that can be the targets of the probes in the new detection devices. The analytes can be, for example, a microbial surface antigen, a specific nucleic acid sequence, an enzyme produced by a specific microorganism, a particular chemical compound, or any material or composition that is associated with

hydrocarbon deposits, such as heavy metals, e.g., chromium, mercury, and copper (Fleck et al., Biotechnology Letters, 22:285-289, 2000).

There are numerous hydrocarbons that can be detected including: ethyl benzene, benzene, cycloheptane, cyclohexane, toluene, methylcyclopentane (MCP), styrene, xylene, biphenyl, jet fuel, gasoline, chlorinated hydrocarbons, polycyclic aromatic hydrocarbons (PAHs), anthracene, pyrene, naphthalene, and of course the many hydrocarbons that make up petroleum, such as methane, butane, propane, ethane, pentane, hexane, heptane, octane, and decane. See also Table II. Based on these hydrocarbons, one of skill in the field can select the appropriate microorganisms (e.g., from Tables I, II, and III) and other materials that serve as targets. Once the targets are selected, one, two, three or more analytes unique to each of those targets are selected, and then probes are prepared that bind specifically to those analytes as described herein.

#### Probe Selection

This section describes the different types of molecules that can be used as probes on different substrates, such as "chips." For any given analyte, there can be one or more types of probes that can be used to specifically bind to the analyte. For example, if the analyte is a nucleic acid molecule, e.g., from a microbe's DNA or RNA, it can be detected using a nucleic acid probe or a protein-based, e.g., polyamide-based probe, such as a zinc-finger binding protein (ZFPs), or a minor groove binder (MGB). If the analyte is a particular antigen, the probe can be an antibody that specifically binds to that antigen.

Substrates can be linked to probes that will detect only nucleic acids (NuScreen™ Chip), only non-nucleic acid (e.g., protein-based or polypeptide-based and other types of analytes such as haptens and chemicals) analytes (ProScreen™ Chips), or both nucleic acid and protein-based analytes (UniScreen™ Chips).

NuScreen™ Chips: These chips are used for analyzing nucleic acid components of a sample. They can analyze DNA, RNA, or both, and do so in their single-stranded or double-stranded form. Probes can be XNA based (DNA, RNA, PNA, LNA, HNA, etc.) or protein and polypeptide based (transcription factors, such as ZFPs, and small molecules, such as MGBs), or a combination of both. XNA probes usually bind to single-stranded nucleic acids, except for triplex forming oligos that bind duplexes. Thus, an optional denaturation step can be involved. Preferred probes are

based on DNA oligonucleotides 16-40 bases long. ZFPs and MGBs recognize nucleic acids in their double-stranded form and thus require no denaturation step. Nucleic acids from a sample can be pre-amplified prior to detection on the NuScreen Chip. Nucleic acids can also be labeled with a detectable moiety during the amplification step. Amplification can be done using conventional techniques such as PCR, Reverse Transcription – Polymerase Chain Reaction (RT-PCR), in vitro transcription (IVT), Nucleic Acid Sequence Based Amplification (NASDA), Rolling Circle Amplification (RCT) etc.

ProScreen™ Chips: These chips are used for analyzing all other components of a mixture that the NuScreen chip cannot detect. Thus, ProScreen chips are used to detect, e.g., proteins, polypeptides, peptides, glycoproteins, antigens, haptens, and small organic molecules. Probes can be protein or peptide based, but can also be, for example, glycoproteins, antigens, haptens, small organic and inorganic molecules, nucleic acid molecules, and aptamers.

UniScreen™ Chips: These chips are universal screening devices, which means that they can detect almost any kind of analyte, be it a specific nucleic acid sequence or a protein or something else, with very high specificity and selectivity. The probes used for detection of all analytes other than nucleic acids are similar to the ones used in ProScreen chip. However, protein and peptide-based nucleic acid probes can be used for detecting nucleic acids, such as DNA and RNA, in the sample. An advantage of protein-based probes, such as ZFP and MGB probes, is that they recognize a specific nucleic acid sequence under physiological conditions, without any requirements to denature the nucleic acids. Thus, they can be combined with probes that are used to detect analytes other than nucleic acids and be effectively used under the same binding conditions.

This invention utilizes the well-known sequence specific recognition properties of certain protein and peptide molecules that bind to nucleic acids selectively. The specific binding reaction does not require denaturation of the nucleic acids and occurs under normal physiological conditions. Specifically, nucleic acid molecules do not need to be denatured to a single-stranded form. ZFPs recognize DNA, RNA, and DNA-RNA duplexes. Binding takes place under physiological reaction conditions and is specific for each ZFP-nucleic acid sequence pair. For additional information on zinc-

finger proteins and their use in arrays, see, e.g., Hanas, WO 99/45388 (published on Sept. 10, 1999); and Wagner et al., WO 00/04382 (published on Jan. 27, 2000).

Thus, for the first time, the new methods enable the simultaneous detection of almost any combination of analytes on the same surface and using the same device, independent of the nature of the analyte. The device that performs such a function is the UniScreen Chip. In one embodiment, biotinylated DNA/RNA target (labeled during PCR/IVT steps) can be used. Labeled nucleic acid targets are captured by ZFPs and detected using anti-biotin antibodies coupled to streptavidin/HRP. In addition, Tyramide Signal Amplification/Rolling Circle Amplification Technology (TSA/RCAT) can be used for further signal amplification. Gold on silver staining methods (similar to immunohistochemical staining techniques) can also be used.

#### Substrate Selection and Methods of Attaching Probes

A variety of different types of substrates (e.g., glass slides) and surfaces can be used to create the new detection devices, and many of different immobilization methods can be used to attach probes to these substrates.

In one embodiment, glass slides are used to prepare biochips. The substrates (such as plates, sheets, films, or membranes) can also be made of silica, silicon, plastic, metal, metal-alloy, inorganic aluminum oxide membranes (e.g., ANOPORE®), and other polymers such as nylon. The surfaces of substrates can be treated with a layer of chemicals prior to attaching probes to enhance the binding or to inhibit non-specific binding during use. For example, glass slides can be coated with self-assembled monolayer (SAM) coatings, such as coatings of aminoalkyl silanes, or of polymeric materials, such as acrylamide and proteins. A variety of commercially available slides can be used. Some examples of such slides include 3D-link® (Surmodics), EZ-Rays® (Mosaic Technologies), Fastslides® (Schleicher and Schuell), Superaldehyde™, and Superamine™ (CEL Technologies).

Probes can be attached covalently to the solid surface of the substrate (but non-covalent attachment methods can also be used). Similar substrate, coating, and attachment chemistries can be used for all three – UniScreen™, ProScreen™, and NuScreen™, but different chemistries are applied.

A number of different chemical surface modifiers can be added to substrates to attach the probes to the substrates. Examples of chemical surface modifiers include N-hydroxy succinimide (NHS) groups, amines, aldehydes, epoxides, carboxyl groups,

hydroxyl groups, hydrazides, hydrophobic groups, membranes, maleimides, biotin, streptavidin, thiol groups, nickel chelates, photoreactive groups, boron groups, thioesters, cysteines (e.g., for native chemical ligation methods of Muir et al., *Proc. Natl. Acad. Sci. USA*, Vol. 95, pp. 6705–6710, June 1998), disulfide groups, alkyl and  
5 acyl halide groups, glutathiones, maltoses, azides, phosphates, and phosphines. Glass slides with such chemically modified surfaces are commercially available for a number of modifications. They can easily be prepared for the rest, using standard methods (Microarray Biochip Technologies, Mark Schena, *Editor*, March 2000, Biotechniques Books).

10 In one embodiment, substrate surfaces reactive towards amines are used. An advantage of this reaction is that it is fast, with no toxic by-products. Examples of such surfaces include NHS-esters, aldehyde, epoxide, acyl halide, and thio-ester. Most proteins, peptides, glycopeptides, etc. have free amine groups, which will react with such surfaces to link them covalently to these surfaces. Nucleic acid probes with  
15 internal or terminal amine groups can also be synthesized, and are commercially available (e.g., from IDT or Operon). Thus, nucleic acids can be bound (e.g., covalently or non-covalently) to surfaces using similar chemistries.

The substrate surfaces need not be reactive towards amines, but many substrate surfaces can be easily converted into amine-reactive substrates with coatings.  
20 Examples of coatings include amine coatings (which can be reacted with bis-NHS cross-linkers and other reagents), thiol coatings (which can be reacted with maleimide-NHS cross-linkers, etc.), gold coatings (which can be reacted with NHS-thiol cross linkers, etc.), streptavidin coatings (which can be reacted with bis-NHS cross-linkers, maleimide-NHS cross-linkers, biotin-NHS cross-linkers, etc.), and BSA coatings  
25 (which can be reacted with bis-NHS cross-linkers, maleimide-NHS cross-linkers, etc.). Alternatively, the probes, rather than the substrate, can be reacted with specific chemical modifiers to make them reactive to the respective surfaces.

A number of other multi-functional cross-linking agents can be used to convert the chemical reactivity of one kind of surface to another. These groups can be  
30 bifunctional, tri-functional, tetra-functional, and so on. Cross-linkers with a number of different functional groups are widely available. Examples of such functional groups include NHS-esters, thio-esters, alkyl halides, acyl halides (e.g., iodoacetamide), thiols, amines, cysteines, histidines, di-sulfides, maleimide, cis-diols, boronic acid,

hydroxamic acid, azides, hydrazines, phosphines, photoreactive groups (e.g., anthraquinone, benzophenone), acrylamide (e.g., acrydite), affinity groups (e.g., biotin, streptavidin, maltose, maltose binding protein, glutathione, glutathione-S-transferase), aldehydes, ketones, carboxylic acids, phosphates, hydrophobic groups (e.g., phenyl, cholesterol), etc. Such cross-linkers can be reacted with the surface or with the probes or with both, to conjugate a probe to a surface.

Other alternatives include thiol reactive surfaces such as acrydite, maleimide, acyl halide and thio-ester surfaces. Such surfaces can covalently link proteins, peptides, glycopeptides, etc., via a (usually present) thiol group. Nucleic acid probes containing pendant thiol-groups can also be easily synthesized.

Alternatively, one can modify glass surfaces with molecules such as polyethylene glycol (PEG). A novel approach to creating such modified surfaces is to use PEGs of mixed lengths (see, e.g., FIGs. 3A and 3B). Exposed ends of PEGs can be activated with bifunctional cross-linkers as mentioned above. As shown in FIG 3B, the varied lengths of PEG linkers create a three-dimensional, rather than a flat, two-dimensional binding environment (FIG. 3A), which provide higher probe attachment densities because of better packing of the biological molecules upon attachment. Packing of biomolecules, such as proteins, would be higher on a slightly three-dimensional or uneven binding surface than on a completely even and flat binding surface.

Yet another alternative is to create a three-dimensional, covalently linked mesh of streptavidin or other linker molecule. The addition of multi-functional cross-linkers, such as NHS-biotin or maleimide-biotin, to this surface regenerates active groups ready for covalently linking (amine- or thio-group containing) probes. The resulting surface is much more reactive with proteins and other probe molecules.

Probes of mixed lengths can also be used to provide an uneven binding environment that can provide higher probe attachment densities because of better packing of the biological molecules upon attachment.

Many other surface modification alternatives (such as photo-crosslinkable surfaces and thermally cross-linkable surfaces) are known to those skilled in the art. Some technologies are commercially available, such as those from Mosaic Technologies (Waltham, MA), Exiqon (Vedbaek, Denmark), Schleicher and Schuell (Keene, NH), Surmodics (St. Paul, MN), Xenopore (Hawthorne, NJ), Pamgene

(Netherlands), Eppendorf (Germany), Prolinx (Bothell, WA), Spectral Genomics (Houston, TX), and Combimatrix (Bothell, WA).

Surfaces other than glass are also suitable for such devices. For example, metallic surfaces, such as gold, silicon, copper, titanium, and aluminum, metal oxides, such as silicon oxide, titanium oxide, and iron oxide, and plastics, such as polystyrene, and polyethylene, zeolites, and other materials can also be used. The devices can also be prepared on LED (Light Emitting Diode) and OLED (Organic Light Emitting Diode) surfaces. An array of LEDs or OLEDs can be used at the base of a probe array. An advantage of such systems is that they provide easy optoelectronic means of result readout. In some cases, the results can be read-out using a naked eye.

Probes can be deposited onto the substrates, e.g., onto a modified surface, using either contact-mode printing methods using solid pins, quill-pins, ink-jet systems, ring-and-pin systems, etc. (see, e.g., U.S. Patents Nos. 6,083,763 and 6,110,426) or non-contact printing methods (using piezoelectric, bubble-jet, syringe, electro-kinetic, mechanical, or acoustic methods. Devices to deposit and distribute probes onto substrate surfaces are produced by, e.g., Packard Instruments. There are many other methods known in the art. Preferred devices for depositing, e.g., spotting, probes onto substrates include solid pins or quill pins (Telechem/ Biorobotics). Each probe can be deposited in one or more replicates to achieve better results. Probes can also be deposited in such a geometric pattern that the read-out can be easily converted to a result by simple visual inspection (see FIGs. 1A-2C). For example, probes can be deposited in a square pattern of nine spots, an "X" pattern of five spots (e.g., FIGs. 1A and 2A), a "V" or "Λ" pattern of three spots (FIG. 1B), or a "+" of five spots (FIG. 1C).

#### Fine-Tuning ("Developing") Devices

After the probes are deposited, the devices/slides/supports are developed using standard techniques according to surface modification and probe attachment chemistries used. For example, NHS-ester activated slides, that have amine-group containing probes attached, can be developed by incubated in a humidity chamber (preferably 75%-80%) and at 4° Celsius for 2-16 hours. The developed slides, in a preferred embodiment, can be kept sealed in an aqueous buffer until the time of their use. The aqueous buffer can also contain blockers such as Bovine Serum Albumin (BSA), milk proteins, Peptidase® (Casein Enzymatic Hydrolysate), glycerol, trehalose

or other such reagents that preserve the activity of attached probes. In other embodiments, the slides can be kept in a dry, cool, and dark environment. An alternative blocking methodology is to deactivate the chip (other than the deposited probes) by irradiating with electromagnetic radiation. This step can be performed after the blocking steps noted above, to denature the blocking agents. This step will reduce the antigenic properties of the surface agents and will result in lower non-specific binding of target molecules during an assay.

### Devices

As shown in FIG. 1A, a detection biochip 20 can contain more than one array 22, in a multi-reaction site (e.g., multispot or multiwell) format, such as sixteen reaction sites 24, such as microwells, on a solid support 26, such as a slide. In the case of a multiwell device, each microwell 24 can contain probes in an array 22 or an "array of arrays" format. Probes can be deposited in an easy to read geometrical pattern (here, a square of nine spots). Each microwell is delimited in that it has partitioned zones. The partitioning can be achieved by chemical treatment or by application of a mask, preferably hydrophobic, onto the surface of the slide, either prior to, or after probe deposition steps. The partitioning can result in the creation of cylindrical microwells that have a higher sample retaining capacity, compared to supports without wells. Each solid support 26 can include scannable markings 28 (such as a bar code) for computer-controlled, automated processing.

FIGs 1A to 1C also illustrate various probe array configurations. FIG. 1A shows probes in an "X" configuration. FIG. 1B shows a "V" or "Λ" configuration, and FIG. 1C shows a "+" configuration.

FIGs. 2A to 2C, show a detection biochip device 40 that contains more than one array 42, in a multi-reaction site (e.g., multiwell) format, such as sixteen reaction sites 44, such as microwells, on a solid support 46, such as a slide. Each microwell 44 can contain probes in an array 42 or an "array of arrays" format. Each microwell 44 is delimited in that it has partitioned zones. Here, partitioning is achieved by applying a mask 43, preferably of a hydrophobic material, onto the surface of the solid support 46, either prior to, or after probe deposition steps. The partitioning creates cylindrical microwells that have a higher sample retaining capacity, compared to supports without wells. The mask 43 can be applied to the support 46 on top of an intermediate organic



layer 45 as shown in the cross-section of FIG. 3B. FIG. 3C shows a three-dimensional view of a single microwell 44.

Each solid support 46 can include a scannable ID marking 48 for computer-controlled, automated processing. Here there are two sets of markings 48, to allow easier access for scanning, or to provide different information on each marking.

One problem with biochip devices is the formation of bubbles during sample application step. An advantage of the cylindrical shape of the wells is that it has fewer problems with bubbles due to curved walls of the microwell. A rectangular shape creates sharp corners, which usually retain air-bubbles. A cylindrical shape does not have that problem.

#### **Mixing Apparatus for Use in Microarrays and Biochips**

Mixing of samples during incubation is important in microarray assays. Binding efficiency of different target analytes to their respective probes is directly linked to their concentration as well as their rate of diffusion. Mixing a sample during incubation helps increase the rate of diffusion thus giving better and more reproducible results. The binding time can also be decreased if efficient means of mixing can be achieved. FIGs. 4A and 4B illustrate solid supports that include micromotors (such as micro-fans and biological motors). These micromotors can be, for example, electric, magnetic, optoelectronic, or biochemical motors. Mixing can also be achieved by incorporating magnetic beads (such as DynaBeads®) in the sample and using a stirrer underneath the biochip to stir each well.

FIG. 4A shows a solid support 76 with sixteen microwells 74. Each microwell 74 is outfitted with a micromotor 73 including microfan blades 75. FIG. 4B shows an enlarged view of a single microwell 74 and one way of attaching the motor to the slide and depositing probe arrays around this motor in four quadrants. Other probe array configurations are possible. Electricity is conveyed by wires 77 that run from one or more electrical connectors 79, e.g., at one end of slide 76, to each micromotor 73. Electricity can also be conveyed by metal or other conductors deposited onto or into the solid support, e.g., using standard printed circuit technology. New miniaturization techniques allow the entire micromotor to be deposited onto the solid support in a similar manner. Slide 76 can include computer-readable markings 78 as described above.

In all of these embodiments, electric motors can be powered using electromagnetic sources such as electronic and photonic means. Some motors can have optically activatable switches that can be switched on using light. Molecules that can be induced to rotate between two geometric shapes, such as *cis*- and *trans*-stilbene that undergo stereo conversion from one to another configuration upon electromagnetic excitation, are one example of such motors. Biological motors, such as ATP synthase, can be powered by electromagnetic sources or by biological reactions. For example, ATP synthase can be attached to a bead (for example, see "Biological machines: from mills to molecules, *Nature Reviews Molecular Cell Biology* 1; 149-152, 2000), and its motor can be attached to another bead. The two beads will rotate with respect to each other, in the presence of molecules such as ATP, and in the process, mix the fluids.

A number of biological molecule based fluid micromixers can be used. For example, these micromixers can be based on ATPase (see, e.g., Soong et al.; *Science*, 290 (5496):1555-1560, 2000; Wang et al., *Nature*, 396:279-282, 1998; Montemagno et al., Nanoscale Biological Engineering and Transport Group, Cornell University, *Nanotechnology*, 10:225-231, 1999). Micromixers can also be based on kinesin, kinesin Related Proteins, myosin, DNA Helicase, and DNA Sliding clamps (see, e.g., Bertram et al., *Journal of Biological Chemistry*, 275(37):28413-28420, 2000; O'Donnell et al., *Journal of Biological Chemistry*, 270(22):13358-13365, 1995; Hingorani et al., *The EMBO Journal*, 18(18):5131-5144, 1999); nucleic acid based rotaxanes and Pseudo-rotaxanes (Ryan et al., *Chemistry and Biology*, 1998); circular triplex forming oligonucleotide (CTFO) and duplex DNA (Rehman et al., 1999); as well as chimeras and derivatives of such proteins and nucleic acids. See also Bishop et al., *Annu. Rev. Biophys. Biomol. Struct.*, 29:577-606, 2000).

Some of these mixing devices can also be incorporated in the surfaces covering biochips, such as cover slips or hybridization chambers. An advantage of such a methodology would be that the mixing apparatus would be compatible with the currently available chip platforms.

### **Novel Hybridization Chambers**

One of the critical issues with current biochip assays is the high variability in results. The new systems can include a novel hybridization chamber that can perform assays on two chips simultaneously and under same reaction conditions. Two biochips

are laid one on top of the other, with the reactive arrays facing each other and separated along the edges with a thin separator. The space left in the middle accommodates the sample, which contacts both of the slides simultaneously. The two slides and the separator may be enclosed in a chamber. Alternatively, a chamber with preformed side protrusions can be designed and two biochips can be inserted to make a reaction chamber. FIGs. 11A to 11G illustrate some embodiments and the components of novel hybridization chambers. The interior chamber of these hybridization modules can be filled in a variety of ways, using a pipet, syringe, or needle.

#### 10 Inverted Array Devices

New inverted array microarray devices are illustrated in FIGs. 5A to 5C and 6A to 6E. These new devices have one or more elevated structures or columns on a solid support or platform. Multiple probes are bound in an array (or an array of arrays) to the surface of the elevated structures. Thus, each elevated structure, denotes a  
15 (multiplexed) reaction site. The device can be used to perform reactions simultaneously or sequentially. Any of the known substrates and chemistries can be used to create such a device, and the elevated structures can be manufactured by various techniques known in the art, such as etching, machining, photolithography, and other microfabrication techniques. Similarly, probes can be attached to the surface of  
20 these devices using a number of different methods as described herein.

FIGs. 5A to 5C show one example of such an inverted array device 90, which can contain more than one array 92, in a multi-reaction site (e.g., multiwell) format, such as sixteen reaction sites 94, such as elevated circular structures, on a solid support 96, such as a slide. Each elevated structure 94 can contain probes in an array 92 or an  
25 "array of arrays" format. Each elevated structure is delimited in that it has partitioned zones, e.g., achieved by application of a mask (or coating) 93, of a hydrophobic or hydrophilic material, onto the surface of slide 96, either prior to, or after probe deposition steps. The partitioning can result in the creation of cylindrical elevated support structures that have a higher sample retaining capacity, compared to supports  
30 without such structures. Each solid support 96 can include scannable markings 98 (such as a bar code) for computer-controlled, automated processing.

FIG. 5B shows device 90 from a cross-sectional side view. FIG. 5C shows two different configurations (cubic and cylindrical) of the elevated structure 94 on solid support 96 in three dimensions.

For some applications, the inverted array devices are surrounded by a liquid barrier or wall, to contain sample fluids introduced onto the surface of the device. In other embodiments, assays are performed using the new inverted array devices by incubating the entire device on or within a chamber, such as a microtiter plate.

FIGs. 6A to 6E illustrate how an inverted array device 110 is inverted and inserted into a microwell or microtiter plate 111. FIG. 6A shows a top view of inverted array device 110 with 96 elevated structures 114 on a solid support 116 having scannable markings 118. FIG. 6B shows a side view of device 110. FIG. 6C shows a top view of a microtiter plate 111 with microwells 113. In use, the inverted array device 110 is inverted and placed onto the microtiter plate 111 to insert each elevated structure 114 into an individual microwell 113. Each microwell contains one sample, and each microwell can contain the same or a different sample. Sets of 2, 3, 4, 5, 10, or more microwells can also contain the same sample.

This device/system is easy to automate, especially with the current robotic systems. The inverted array system/device can easily be moved from one reaction vessel to another. Each device can optionally include a "handle" to help move the device, or a robotic arm can move the device using standard devices and techniques if no handle is provided.

### **Three-Dimensional Porous Array Devices**

New three-dimensional porous microarray devices are illustrated in FIGs. 7A to 7C and 8A to 8C. These new devices consist of one or more porous gel-bound probes in an array or an array of arrays format. Each device denotes a (multiplexed) reaction site. Any of the known substrates and chemistries can be used to create such a device (see. e.g., Stillman BA, Tonkinson JL, Schleicher and Schuell; *Biotechniques*, 29(3), 630-635, 2000; Rehmna et. al; Mosaic Technologies Inc., *Nucleic Acids Research*, 27(2), 649-655, 1999).

The new devices can be manufactured in a number of ways. In one implementation, small holes are manufactured in a solid three-dimensional object using photolithography, etching, drilling, or other techniques known in the art (FIGs. 7A and

8A). The holes can be of any geometric shape and can also have slits or grooves. Probes can be immobilized in these holes using a variety of methods including embedding them in a polymeric matrix. The probes can be separately mixed with a pre-polymeric gel and poured into or dispensed or deposited into each of the different  
25 holes to create polymeric, porous plugs (FIGs. 7C and 8B). Subsequently, the probe-gel mixture can be polymerized using photo-initiators or other methods known in the art (see, e.g., Arenkov et. al; *Analytical Biochemistry*, 278, 123-131, 2000; Timofeev et. al; *Nucleic Acids Research*, 24 (16), 3142-3148, 1996; Mirzabekov et al.; *Methods in Molecular Biology*, 170, 17-38, 2001; and Mirzabekov et al.; U.S. Patent No.  
10 5,981,734, Nov. 9, 1999). The polymerized probe-gel material can be secured in the three-dimensional substrate by application of a secondary mask (FIG. 7A) or a membrane on either or both sides (FIG. 8C). The securing material can also have slits or holes. Dumb-bell shaped polymeric plugs can also be used to immobilize probes, especially when the holes have grooves on the outside (FIG. 8B) to help prevent the  
15 plugs from slipping or falling out. Such holes and plugs can further be sealed in from two sides with another membrane with or without slits. The final three-dimensional probe array device can be round, e.g., in the shape of a microwell or petri dish (FIG. 7B).

There are a number of advantages of this type of device including:

20 (i) The probes are bound in a three-dimensional porous material. Three-dimensional probe spots result in a higher amount and concentration of the probe bound to a spot, compared to a two-dimensional spot (i.e., binding of probes to a flat surface). This corresponds to increased spot resolution of the scanned microarray.

(ii) Porosity of the spot results in a porous array of spots. This gives better  
25 binding performance, due to enhanced diffusion of the target material through the entire spot as well as around the array of spots.

(iii) The diffusion properties of the new devices can be further improved by mechanically mixing the fluid in a direction orthogonal to the plane of the array. For example, sample mixing or transport can also be achieved or increased by using  
30 electrophoresis and other electronic (e.g., Nanogen, U.S. Patent No. 6,238,624) or optical means.

(iv) Since the assay time is substantially reduced, this type of device can be adapted to manufacture a point-of-care device as well, especially where a single

biochip is placed in a container and the fluid mixing is mechanically controlled (See, FIGs. 7A-C).

(v) Post-assay detection of the array results can be simplified and automated because the position and the size of each probe spot is uniform. This results in lower spot-to-spot and array-to-array variation.

(vi) Almost any material can be used as a substrate, since the problem of background fluorescence of the material is completely eliminated. The auto-fluorescence of only the probe-gel mixture needs to be considered.

(vii) Almost any attachment chemistry can be used to attach probes to the porous material. The attachment can be covalent as well as non-covalent. Thus, this type of format offers a wide variety of choices.

(viii) Adopting this type of a design for the microarray devices will substantially reduce manufacturing costs.

Additional references of interest include: U.S. Patent No. 5,843,767 and websites on the Internet for [homer.hsr.ornl.gov/cbps/Genosensors.htm](http://homer.hsr.ornl.gov/cbps/Genosensors.htm).

### **Microfluidic Concentrators**

New microfluidics-based devices (for example, see Chou et al., *Proc. Solid State Act. Sensor Workshop*, Hilton Head, S.C., 2000) can also be incorporated into the biochip devices or be used separately. Such microfluidic devices can have chambers or channels that each contain an array of probes that bind complementary analyte targets. This type of an array serves to concentrate related analytes onto a single spot. Bound analytes could be released, using labile linkers on probes, and directed into a second channel or chamber. There the analytes could be further analyzed either on a second array of probes or in a capillary electrophoretic channel. Thus, each analyte is analyzed in two orthogonal dimensions providing a more accurate result. The microfluidic devices can be made on glass, polymers, plastic, silicon, metals, and a variety of other solid substrates.

As shown for example FIG. 9, a device has a central chamber 130 including a set of probes, which are placed at the intersection of orthogonal arteries 132 and 134 feeding into other chambers. The probes attached at this nodal point are non-specific in that they bind to a set of target molecules that are unique and yet have at least one similar characteristic. In a way, these nodes act at points in the stream where similar

analytes are concentrated. Once this part of the assay is complete, the orthogonal array is activated and transports each concentrated target set into separate chambers 136 where they are further analyzed into unique positions, based on a second set of interactions. Thus, this system incorporates two orthogonal detection probes and will thus have a much better detection capability. Another advantage of this type of system, besides being a fast and improved detector, is that it can be combined with other types of biochip devices for enhancing their performance.

### Methods of Using Hydrocarbon Detection Devices

The new detection devices, e.g., oil prospecting kits, are simple to use by technicians, engineers, surveyors, and/or agricultural workers. Samples from the environment (e.g., soil, sand, clay, and water) are easy to obtain and apply to the detection kits. The results are easily read from a detection kit using a standard reader device, e.g., a device that reads fluorescent light or other signals emitted from the probes of the detection kit, or from antibodies bound to analytes that are bound to probes on a substrate (e.g., in a sandwich assay format).

Specimen collection and purification methods (for subsequent association to a probe array of the system/device) include all front-end processes such as biological specimen collection, purification, isolation, and labeling as required. These protocols are standard, published, and are known to those skilled in the art.

One method of extracting and purifying microbial DNA from soil, clay, or sand is modified from that described by Guo, et al. (Hybridization Analysis of Microbial DNA from Fuel Oil-Contaminated and Noncontaminated Soil. Microb Ecol 34:178-187, 1997).

Briefly, several boreholes are drilled, e.g., 10 feet apart. Soil samples are taken from these boreholes at set depths, e.g., from about 4 to 6 feet. The samples are placed on ice immediately following collection and transported to a laboratory for strain isolation or storage at  $-20^{\circ}\text{C}$  for microbial DNA extraction.

Before extracting microbial DNA, extracellular DNA in the sample is removed. An amount, e.g., one to five grams, of soil is washed, e.g., with a sodium phosphate buffer through vortexing-and-centrifugation steps. Microbes in the soil sample are then lysed, e.g., with the standard repeated freezing-thawing method. The total DNA is extracted with an alcohol, e.g., with  $\text{CHCl}_3$ :isoamyl alcohol, and precipitated, e.g., with

13% polyethylene glycol. The precipitate is washed, e.g., with 70% cold ethanol, and air-dried. A crude DNA extract, obtained above, can be further purified by precipitating with ammonium acetate and passing through a Magic Minipreps Column (Promega Co., Madison, Wis.).

- 5 Similar methods can be used to extract RNA, and even simpler methods can be used to extract intact microorganisms if surface antigens are the target analytes. Other standard techniques can be used to isolate microbial enzymes that are indicators of the presence of hydrocarbons in the environment.

Many assay methods are available and are known to those skilled in this field.

- 10 NuScreen assays will be based on nucleic acid detection by hybridization. Samples would be put on the multiplexed test sites and incubated for binding to occur. The single base extension (SBE) method, with the queried base as the last nucleotide of the probe oligo, can be used for polymorphism analyses. Chemical as well as enzymatic ligation methods, as well as rolling circle amplification, can also be used. Reagents for
- 15 performing SBE will be added and the test chamber will be sealed. The test sample will be washed with a large volume of SSC or other aqueous washing solutions to remove non-specific binding from the surface of the array.

- If non-fluorescent nucleotides are used in the SBE reaction, they will be developed using a secondary molecule labeled with a fluorophore (for example, a
- 20 fluorescent streptavidin/antibody, or an HRP-streptavidin/antibody conjugate or an EFL-utilizing molecule-antibody conjugate or gold-antibody conjugate with subsequent silver treatment etc.). In one detection method, DNA/RNA is labeled with biotinylated nucleotides during PCR/IVT. The Tyramide Signal Amplification (TSA) protocol can also be used for detection (from NEN). RCAT (Molecular staging/Amersham) can be
- 25 used in place of TSA for signal amplification. Such techniques and reagents are widely known and commercially available. A final washing step with an aqueous solution is used to remove unused fluorophores, unbound antibodies, and any other reporter groups, etc.

- ProScreen™ assays will include modified Western blot, ELISA, and related
- 30 methods, primarily for protein, peptides, nucleic acids and other biological moieties, (competition assays and others can also be used). Samples are loaded on the multiplexed test sites and incubated for a few minutes to several hours for binding to occur. Concentration of the probes on the biochip will be optimized according to the



binding affinity of various biomolecules to their corresponding probes. Nucleic acid components of the test sample can be amplified and labeled (with fluorophores, etc.) separately prior to the biochip assay. The amplified and labeled nucleic acid fraction can be combined with the non-nucleic acid fraction, and then applied to the microarray.

- 5 Subsequent to the binding reaction, the test sample will be washed away with a large volume of phosphate buffered saline (PBS) or another aqueous washing solution. This will also remove non-specific binder from the surface of the chip.

- Binding reactions will be developed using secondary molecules labeled with a reporter group, such as a fluorophore (for example, a fluorescent antibody, or a  
10 horse-radish peroxidase (HRP)-antibody conjugate) utilizing molecule-antibody conjugate or gold-antibody conjugate with subsequent silver treatment. In one useful method, sandwich ELISA coupled to biotin/HRP is used. A TSA step can be used as signal amplification method. Rolling Circle Amplification Technology (RCAT; Molecular staging/Amersham) can be used in place of TSA for signal amplification.
- 15 Alternatively, a chemiluminescent, radioactive, electroactive, redox active, or IR-active agent is used. Such techniques and reagents are widely known and commercially available. Again, a final washing step with an aqueous solution is used to remove unused fluorophores etc.

- The results are monitored by scanning and/or imaging of the detection kit, for  
20 locating the association (binding/hybridization/extension) of the target molecules/agents with specific sites in the arrays (within each device). Such methods are widely used and devices to perform these operations are commercially available. Examples include the GenePix™ system (Axon Instruments, Union City, CA), Scanarray™ (Packard BioSciences, MA), and Arrayworx™ (Applied Precision, WA).

- 25 The results are determined by processing the images to determine information about the target biological sample such as the presence and amount of specific molecular/other constituents that leads to the screening output. Various tools are used to determine the results (and the names of the hydrocarbons present in the sample) from the read-out of any test slide. Commercially available softwares such as GenePix  
30 Pro™ (Axon Instruments), Scanarray™ (Packard), Microsoft® Excel® (Microsoft), and Adobe® Photoshop® (Adobe) can be used, e.g., with minor modifications.

## EXAMPLES

The invention is further described in the following examples, which do not limit the scope of the invention described in the claims.

5

### Example 1: Preparation of Clustered Probe Arrays on Various Solid Supports

A blank 3D-Link™ slide (Surmodics), which contain NHS-ester groups at the surface of the slides, is used in this example. The slide is placed in a chamber for arraying of probes onto the slide surface. Probe molecules (proteins, such as  
10 antibodies, ZFPs, peptides, MGBs, amine-labeled nucleic acids and other probe molecules) are dissolved in buffers at slightly basic pH (for example, bicarbonate buffer at pH 8.3) and are spotted onto the glass slides using an arrayer. All the probe molecules contain an amine group for reaction with the slide surface. The spotting solution can also contain chemicals that have low vapor pressure (high boiling point)  
15 and that preserve the activity of probe molecules (such as glycerol, trehalose, and polyethylene glycol). The spotting is done under controlled conditions (such as humidity, in one instance around 70% relative humidity, temperature, for example around 4°C., pressure, and air-flow).

After the probes are spotted, the slides are put in the development chamber for  
20 1-12 hours. The chamber is kept under controlled conditions (such as humidity, temperature, pressure, and air-flow) as well. The slides are then treated with a blocking buffer (aqueous buffer, pH 8.3 containing BSA or other blocking reagents) for an appropriate amount of time. The slides are then washed and stored.

A blank EZ-Ray® slide (Mosaic Technologies) is treated with an aqueous buffer  
25 containing reducing agents (such as DTT or TCEP) to activate the slide surface into free thiol form. The reducing agents are then washed away with water and the activated slide can be stored under inert atmosphere in a cool, dark place. Activated thiol-surface is next converted to an amine reactive surface. The slide is treated with a hetero-bifunctional cross-linkers, such as N-succinimidyl-3-maleimidopropionate  
30 (SMP), N-(11-Maleimido-undecanoyloxy)-sulfo-succinimide (Sulfo-KMUS) (or similar agents that contain a thiol-reactive maleimide group on one end and an amine-reactive NHS-ester group at the other), in aqueous buffer and at neutral pH (other reaction conditions, such as a different pH, can also be used). Thiols react with the

maleimide moieties of the cross-linker, thus converting thiol-groups into amine-reactive NHS-ester groups. The slide is placed in a chamber for arraying of probes onto the slide surface. Probe molecules (proteins, such as antibodies, antigens and ZFPs, peptides, such as antigens, haptens and MGBs, glycoproteins, polysaccharides, amine-  
5 labeled nucleic acids and other probe molecules) are dissolved in buffers at slightly basic pH (for example, bicarbonate buffer at pH 8.3) and are spotted onto the glass slides using an arrayer. All the probe molecules contain an amine group for reaction with the slide surface. Spotting solution can also contain chemicals that have low  
10 vapor pressure (high boiling point) and that preserve the activity of probe molecules (such as glycerol, trehalose, and polyethylene glycol). The spotting is done under controlled conditions (such as humidity, in one instance around 70% relative humidity, temperature, for example around 4°C., pressure, and air-flow).

After the probes are spotted, the slides are put in the development chamber for 1-12 hours. The chamber is kept under controlled conditions (such as humidity,  
15 temperature, pressure, air-flow etc.) as well. The slides are then treated with a blocking buffer (aqueous buffer, pH 8.3 containing BSA or other blocking reagents) for an appropriate amount of time, washed, and stored.

Xenoslide A™ (aminosilane slides from Xenopore) slides are silanated and ready to use as received. They can be stored at room temperature. A solution of the  
20 probes (nucleic acids, proteins, etc.) is prepared to be spotted. The DNA concentration can be in the range of 1 ng to 1 µg per ml. Spot size can be controlled by use of solvent mixtures. Correct choice of co-solvent will result in lower surface tension of the mixture compared to water and controlled spreading of the spot. Volatility of the solvent mixture and thus the drying time can also be controlled by solvent composition.  
25 Use of a lower volatility co-solvent will increase the drying time. DMSO is frequently used since it is a good solvent for DNA, is miscible with water in all proportions, and has lower surface tension and lower volatility than water. Typically, up to 50% DMSO is used. Alternatively, glycerol can be used in place of DMSO. The solution is spotted onto the slide. If using only water, it is helpful to maintain a humidity of 75-80% for a  
30 few minutes to allow binding to take place. DNA can be cross-linked to the slide by exposing the slide to UV light with up to about 200 millijoules of radiation. The slide is now ready for hybridization.

UniScreen™ Biochips can be prepared as follows. Microscope glass slides with thiols on the surface (such as EZ-Ray™ slides from Mosaic, or Thiol slides from Xenopore) are activated to a free thiol form, and are then washed with water and stored under nitrogen atmosphere in a cool, dark place. The slides are treated with hetero-  
5 bifunctional cross-linkers, such as N-succinimidyl-3-maleimidopropionate (SMP), N-(11-Maleimido-undecanoyloxy)-sulfo-succinimide (Sulfo-KMUS) etc., dissolved in aqueous buffers at neutral pH. The thiols react with the maleimide moieties of the cross-linker, converting them into amine-reactive compounds. Probe molecules (antibodies, antigens, ZFPs, MGBs, amine-labeled nucleic acids) are dissolved in  
10 buffers with slightly basic pH (for example, bicarbonate buffer at pH 8.3) and are spotted onto the glass slides using an arrayer. The spotting is done under controlled humidity (around 70% relative humidity) and temperature (around 16°C.) conditions.

After the probes are spotted, the slides are put into the development chamber for 1-12 hours. The chamber is kept under controlled conditions (such as 70% RH, 16°C.).  
15 The slides are then treated with a blocking buffer (aqueous buffer, pH 8.3 containing BSA and other reagents) for an appropriate amount of time, and are then washed and stored.

#### **Example 2: Petroleum-Prospecting Kit**

20 Oil exploration and prospecting requires sensitive surface analysis methods to detect low molecular weight hydrocarbon seepages and concentrations (see, e.g., Wagner et al., MicroPro GmbH.; Microbial Prospection for Oil and Gas Onshore and Offshore in North-West Europe. 2000. The increased hydrocarbon supply above the oil fields creates ideal conditions for development and growth of a select species of  
25 microorganisms.

The microorganisms listed in Table IV are used as indicators for the presence of petroleum in the vicinity of a soil sample.

**Table IV**

	<b>Benzene:</b> <i>Pseudomonas aeruginosa</i> , <i>P. alcaligenes</i> , <i>P. pickettii</i> , <i>P. putida</i> , <i>Alcaligenes faecalis</i> , <i>P. paucimobilis</i> , <i>P. stutzeri</i> , <i>P. fluorescens</i>
5	<b>TMB (1,2,4-trimethylbenzene):</b> <i>Pseudomonas aeruginosa</i> , <i>P. stutzeri</i> , <i>P. alcaligenes</i> , <i>P. pickettii</i> , <i>P. putida</i> , <i>Alcaligenes faecalis</i>
	<b>Ethylbenzene:</b> <i>P. stutzeri</i> , <i>P. alcaligenes</i> , <i>P. pickettii</i> , <i>P. paucimobilis</i> , <i>P. fluorescens</i> , <i>Alcaligenes faecalis</i>
	<b>Naphthalene:</b> <i>Pseudomonas aeruginosa</i> , <i>P. stutzeri</i> , <i>P. alcaligenes</i> , <i>P. pickettii</i> , <i>P. putida</i> , 10 <i>Alcaligenes faecalis</i>
	<b>Hexane:</b> <i>P. stutzeri</i> , <i>P. alcaligenes</i> , <i>P. pickettii</i> , <i>P. paucimobilis</i> , <i>P. putida</i> , <i>Alcaligenes faecalis</i>
	<b>Octane:</b> <i>Pseudomonas aeruginosa</i> , <i>P. stutzeri</i> , <i>P. alcaligenes</i> , <i>P. pickettii</i> , <i>P. fluorescens</i> , 15 <i>Alcaligenes faecalis</i>
	<b>Decane:</b> <i>Pseudomonas aeruginosa</i> , <i>P. stutzeri</i>
	<b>TMP (2,2,4-trimethylpentane):</b> <i>Pseudomonas aeruginosa</i> , <i>P. stutzeri</i> , <i>P. alcaligenes</i> , <i>P. pickettii</i> , <i>Alcaligenes faecalis</i>
	<b>MBT (2-methylbutane):</b> <i>P. stutzeri</i> , <i>P. alcaligenes</i> , <i>P. pickettii</i> , <i>P. paucimobilis</i> , <i>P. fluorescens</i> , <i>Alcaligenes faecalis</i>
	<b>Cyclohexane:</b> <i>Pseudomonas aeruginosa</i> , <i>P. stutzeri</i> , <i>P. alcaligenes</i> , <i>P. pickettii</i> , <i>P. paucimobilis</i> , <i>P. fluorescens</i> , <i>Alcaligenes faecalis</i>
20	<b>Cycloheptane:</b> <i>Pseudomonas aeruginosa</i> , <i>P. stutzeri</i> , <i>P. alcaligenes</i> , <i>P. pickettii</i> , <i>P. paucimobilis</i> , <i>P. putida</i> , <i>P. fluorescens</i> , <i>Alcaligenes faecalis</i>
	<b>MCP (methylcyclopentane):</b> <i>Pseudomonas aeruginosa</i> , <i>P. stutzeri</i> , <i>P. alcaligenes</i> , <i>P. pickettii</i> , 25 <i>Alcaligenes faecalis</i>

A biochip based test kit is prepared to detect the presence or absence of such microorganisms in soil samples from potential oil exploration sites by selecting one or more analytes, surface antigens, that are unique to this class of microorganisms. The selected surface antigens are then used to prepare monoclonal antibodies that bind to these antigens using standard techniques. The monoclonal antibodies are tested to confirm that they bind specifically to these surface antigens, and are then bound to a glass slide in a 16-microwell format.

**Example 3: Polycyclic Aromatic Hydrocarbon Detection Kit**

Polycyclic aromatic hydrocarbon (PAH) degrading bacteria have adapted metabolisms that oxidize low gaseous hydrocarbons. Table V lists microorganisms that are indicators of the presence of PAHs in the vicinity of a soil sample.

**Table V Polycyclic Aromatic Hydrocarbons (PAHs)**

	<i>Sphingomonas aromaticivorans</i> F199
	<i>Sphingomonas yanoikuyae</i> B1
10	<b>Phenanthrene:</b>
	<i>Mycobacterium</i> sp. strain 'FSPHF'
	<i>Pseudomonas putida</i> strains PB4 'Dw 2-21'
	<i>Sphingomonas paucimobilis</i> strains (DSMZ 6900), TB4 and 'PF I'
15	<i>Sphingomonas</i> sp. strains RP003, RP006, WP01
	<i>Xanthomonas maltophila</i> strain 'MK Phe'
	<b>Anthracene:</b>
	<i>Mycobacterium</i> sp. stain 'FFSPH'
	<i>Pseudomonas</i> sp. strain (NCIMB 12229)
20	<i>Sphingomonas paucimobilis</i> strain 'Ba II'
	<i>Xanthomonas maltophila</i> strains 'MK Anth I', and 'MK Anth II'
	<b>Pyrene:</b>
	<i>Gordona</i> sp. strain 'bp9'
	<i>Mycobacterium gilvum</i> strain (DSMZ 9487)
25	<i>Rhodococcus</i> sp. strain 'UW I'
	<b>Fluoranthene:</b>
	<i>Mycobacterium</i> sp. strain 'FFSPH'
	<i>Sphingomonas paucimobilis</i> strains 'PF I', EPA505
	<b>Naphthalene:</b>
30	<i>Alcaligenes faecalis</i>
	<i>Pseudomonas aeruginosa</i>
	<i>Pseudomonas alcaligenes</i>
	<i>Pseudomonas fluorescens</i> strains (DSMZ 6505)
35	<i>Pseudomonas putida</i> strains PaW 736 (DSMZ 4302/NCIB 9816), PpG7 (DSMZ 4476), (DSMZ 50222), (DSMZ 50208), and ATCC17484
	<i>Pseudomonas stutzeri</i>
	<i>Pseudomonas pickettii</i>
	<i>Pseudomonas</i> sp strains 'MK Naph I', 'MK Naph II', 'MK Naph III', ATCC17483,
40	<i>Sphingomonas paucimobilis</i> strain (DSMZ 6900)
	<i>Xanthomonas maltophila</i> strains 'MK Phe', 'MK Anth I', and 'MK Anth II'

A biochip based test kit is prepared to detect the presence or absence of such microorganisms in soil samples from potential PAH sites by selecting one or more analytes, regions of 16S ribosomal RNA, that are unique to this class of microorganisms. The selected 16S ribosomal RNA regions are then used to prepare 25-mer ribonucleic acid molecule probes that hybridize specifically to these RNAs using standard techniques. The probes are tested to confirm that they bind specifically to

these target microorganisms, and are then bound to a glass slide in a 96 microwell format.

Alternatively, the probes for the 16S rRNA analytes can be designed to be specific for each separate species of bacteria or fungi, thereby providing a test kit that can distinguish between different PAHs in a sample.

#### **Example 4: Methane Detection Kit**

Methanotrophic bacteria have adapted metabolisms that oxidize methane as their sole source of carbon and energy (see, e.g., Wrenn B, Venosa A; Selective enumeration of aromatic and aliphatic hydrocarbon degrading bacteria by a most probable number procedure, *Canadian Journal of Microbiology*, 42 (3), 242-248, 1996). Specific methanotrophic bacteria are listed in Table III.

A biochip based test kit can be prepared to detect the presence or absence of such microorganisms in soil samples from test sites. The specific gene *pmoA* serves as an accurate marker for the presence of methane metabolizing bacteria, and thus, the presence of methane in a sample. A ZFP that binds specifically to a target region in the *pmoA* gene is designed, and multiple ZFP probes are bound to a glass slide.

In addition to the ZFP probes, monoclonal antibodies that bind to specific surface antigens that are unique to each of the 11 microorganisms listed in Table III are also bound to the glass slide. Groups of antibodies that bind specifically to one microorganism are deposited in each well of the slide. Thus, each well contains monoclonal antibodies that bind only to one methanotroph. Only when at least 5 of the 11 antibody wells, as well as the ZFP wells, indicate the presence of a methanotroph will the overall result be considered positive.

**Example 5: Toluene Detection Kit**

The following microorganisms are indicators of the presence of toluene in a sample.

5        *Alcaligenes faecalis*, *Pseudomonas aeruginosa*, *Pseudomonas alcaligenes*,  
         *Pseudomonas fluorescens*, *Pseudomonas paucimobilis*, *Pseudomonas pickettii*,  
         *Pseudomonas putida* strains F1, mt2 PaW1 (DSMZ 3931), mt 14-26 (DSMZ 3934),  
         *Pseudomonas stutzeri*, *Ralstonia sp.* strain PHS1, *Stenotrophomonas maltophilia* strain  
10        T3-c

10        A biochip based test kit is prepared to detect the presence or absence of such  
microorganisms in soil samples from potential toluene sites by selecting one or more  
analytes, regions of 16S ribosomal RNA, that are each unique to each of the  
microorganisms in this list. The selected 16S ribosomal RNA regions are then used to  
15        prepare 25-mer ribonucleic acid molecule probes that hybridize specifically to these  
RNAs using standard techniques. The ribonucleic acid molecule probes are tested to  
confirm that they bind specifically to their respective target microorganisms, and are  
then bound to a glass slide in a 96 microwell format.

20        **Example 6: Jet Fuel Detection Kit**

         A biochip based test kit is prepared to detect the presence or absence of  
*Sphingomonas sp.* strain ANT23 and strain ANT17 in soil samples from potential jet  
fuel contaminated sites by selecting one or more analytes, regions of 16S ribosomal  
RNA, that are each unique to each of these two strains. The selected 16S ribosomal  
25        RNA regions are then used to prepare 25-mer ribonucleic acid molecule probes that  
hybridize specifically to these RNAs using standard techniques. The ribonucleic acid  
molecule probes are tested to confirm that they bind specifically to their respective  
target microorganisms, and are then bound to a glass slide in a 96 microwell format.

         In addition, monoclonal antibodies that bind specifically to surface antigens on  
30        each of these two strains are bound to the same glass slide. Only when at both the  
antibodies and the ribonucleic acid probes indicate the presence of a *Sphingomonas*  
will the overall result be considered positive.



**OTHER EMBODIMENTS**

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate  
5 and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

**WHAT IS CLAIMED IS:**

1. A method of detecting a hydrocarbon in a sample, the method comprising:
  - (a) obtaining a sample;
  - (b) obtaining an array of probes on a substrate, wherein the probes specifically  
5 bind to a plurality of analytes of one or more targets associated with the hydrocarbon,  
and wherein there are at least two different types of analytes for each target;
  - (c) contacting the sample to the probes in the array under conditions that enable  
the probes to specifically bind to any analytes in the sample to form complexes; and
  - (d) detecting complexes on the array;  
10 wherein the presence of a complex indicates the presence of the hydrocarbon in  
the sample.
2. The method of claim 1, wherein the sample comprises soil, sand, or water.
3. The method of claim 1, wherein there are at least two targets associated with  
the hydrocarbon.
- 15 4. The method of claim 1, wherein the method is used to detect multiple  
hydrocarbons in the sample.
5. The method of claim 1, wherein the targets are microorganisms.
6. The method of claim 1, wherein the targets are bacteria or fungi.
7. The method of claim 1, wherein the at least two different types of analytes  
20 for each target are a nucleic acid molecule and a surface antigen specific for the target.
8. The method of claim 1, wherein the at least two different types of analytes  
for each target are a ribosomal ribonucleic acid (rRNA) molecule and a surface antigen  
specific for the target.
9. The method of claim 1, wherein the at least two different types of analytes  
25 for each target are a ribosomal ribonucleic acid (rRNA) molecule and a gene that  
encodes an enzyme involved in metabolism of the hydrocarbon of the target.

10. The method of claim 7, wherein the nucleic acid molecule is the pmoA gene.

11. The method of claim 1, wherein the hydrocarbon is listed in Table II.

12. The method of claim 1, wherein the hydrocarbon is petroleum.

5 13. The method of claim 1, wherein the hydrocarbon is a polycyclic aromatic hydrocarbon, toluene, benzene, or a chlorinated hydrocarbon.

14. The method of claim 1, wherein the target is a microorganism listed in Table II.

10 15. The method of claim 1, wherein the hydrocarbon is petroleum, and the targets are any one or more of the microorganisms listed in Table IV.

16. The method of claim 1, wherein the hydrocarbon is a polycyclic aromatic hydrocarbon (PAH), and the targets are any one or more of the microorganisms listed in Table V.

15 17. The method of claim 1, wherein the hydrocarbon is toluene, and the targets are any one or more of: *Alcaligenes faecalis*, *Pseudomonas aeruginosa*, *Pseudomonas alcaligenes*, *Pseudomonas fluorescens*, *Pseudomonas paucimobilis*, *Pseudomonas pickettii*, *Pseudomonas putida* strains F1, mt2 PaW1 (DSMZ 3931), mt 14-26 (DSMZ 3934), *Pseudomonas stutzeri*, *Ralstonia sp.* strain PHS1, or *Stenotrophomonas maltophilia* strain T3-c.

20 18. The method of claim 1, wherein the hydrocarbon is jet fuel, and the targets are any one or more of: *Sphingomonas sp.* strain ANT23 and strain ANT17.

19. The method of claim 1, wherein the hydrocarbon is gasoline, and the targets are any one or more of: *Pseudomonas aeruginosa*, *P. stutzeri*, *P. alcaligenes*, *P. pickettii*, *P. paucimobilis*, *P. putida*, *P. fluorescens*, *Alcaligenes faecalis*

25 20. A method of detecting petroleum in a sample, the method comprising:  
(a) obtaining a sample;  
(b) obtaining an array of probes on a substrate, wherein the probes specifically

bind to analytes of at least ten different microorganisms associated with petroleum, wherein there are at least two different types of analytes for each microorganism, and wherein the microorganisms are selected from Table II or Table IV;

(c) contacting the sample to the probes in the array under conditions that enable the probes to specifically bind to any analytes in the sample to form complexes; and

(d) detecting complexes on the array;

wherein the presence of a complex indicates the presence of petroleum in the sample.

21. The method of claim 20, wherein the microorganisms are listed in Table IV.

22. The method of claim 20, wherein the probes specifically bind to analytes of at least twenty different microorganisms.

23. The method of claim 20, wherein the sample comprises soil, sand, or water.

24. The method of claim 20, wherein the at least two different types of analytes for each microorganism are a nucleic acid molecule and a surface antigen specific for the microorganism.

25. The method of claim 1, wherein the at least two different types of analytes for each microorganism are a ribosomal ribonucleic acid (rRNA) molecule and a surface antigen specific for the microorganism.

26. The method of claim 1, wherein the at least two different types of analytes for each microorganism are a ribosomal ribonucleic acid (rRNA) molecule and a gene that encodes an enzyme involved in metabolism of a petroleum hydrocarbon, and wherein the rRNA molecule is specific for the microorganism.

27. The method of claim 20, wherein the array of probes comprises at least twenty different probes, each probe specifically binding to a different one of the at least two different analytes for each of the at least ten different microorganism.

28. The method of claim 20, wherein the microorganisms are any ten or more of: *Pseudomonas aeruginosa*, *P. stutzeri*, *P. alcaligenes*, *P. pickettii*, *P. fluorescens*, *P. putida*, *P. paucimobilis*, *Alcaligenes faecalis*, *Agrobacterium rubi*, *Alcaligenes faecalis*,  
5 *Alcaligenes sp.*, *Arthrobacter oxydans*, *Azospirillum sp.*, *Bacillus macroides*, *Bordetella parapertussis*, *Microbacterium lacticum*, *Microbacterium laevaniformans*, *P. agarici*, *P. corrugata*, *P. pseudoalcaligenes*, *P. syringae*, *Ralstonia sp.* strain PHS1, *Rhodococcus marinoascens*, *Sphingomonas yanoikuyae*, *Stenotrophomonas maltophilia*.

10

29. The method of claim 28, wherein twenty or more of the microorganisms are selected.

30. A device for detecting a hydrocarbon comprising:

15 (a) a substrate having a surface;

(b) an array of probes bound to the surface, wherein the probes specifically bind to a plurality of analytes of ten or more targets associated with the hydrocarbon, wherein there are at least two different types of analytes for each target, and wherein the array of probes comprises at least twenty different probes, each probe specifically  
20 binding to a different one of the at least two different analytes for each of the at least ten different targets.

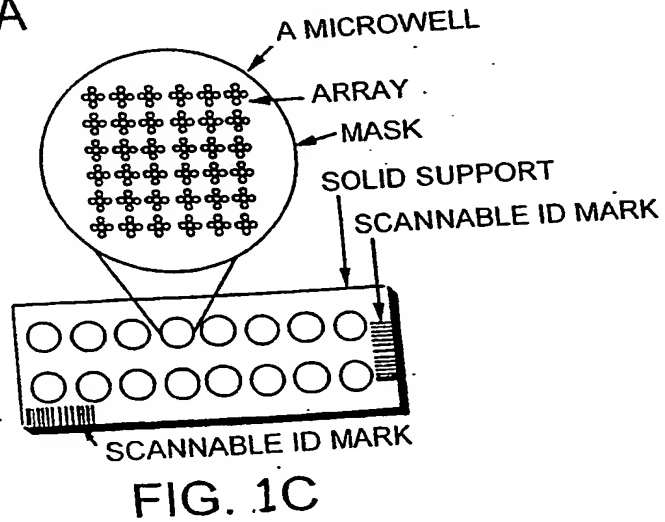
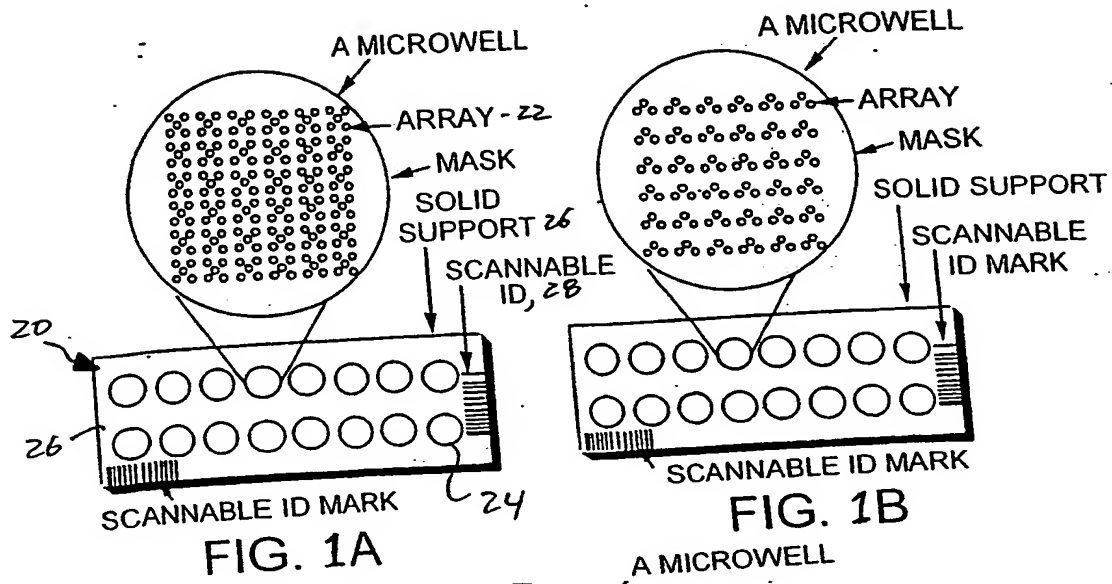
31. The device of claim 30, wherein the targets are microorganisms.

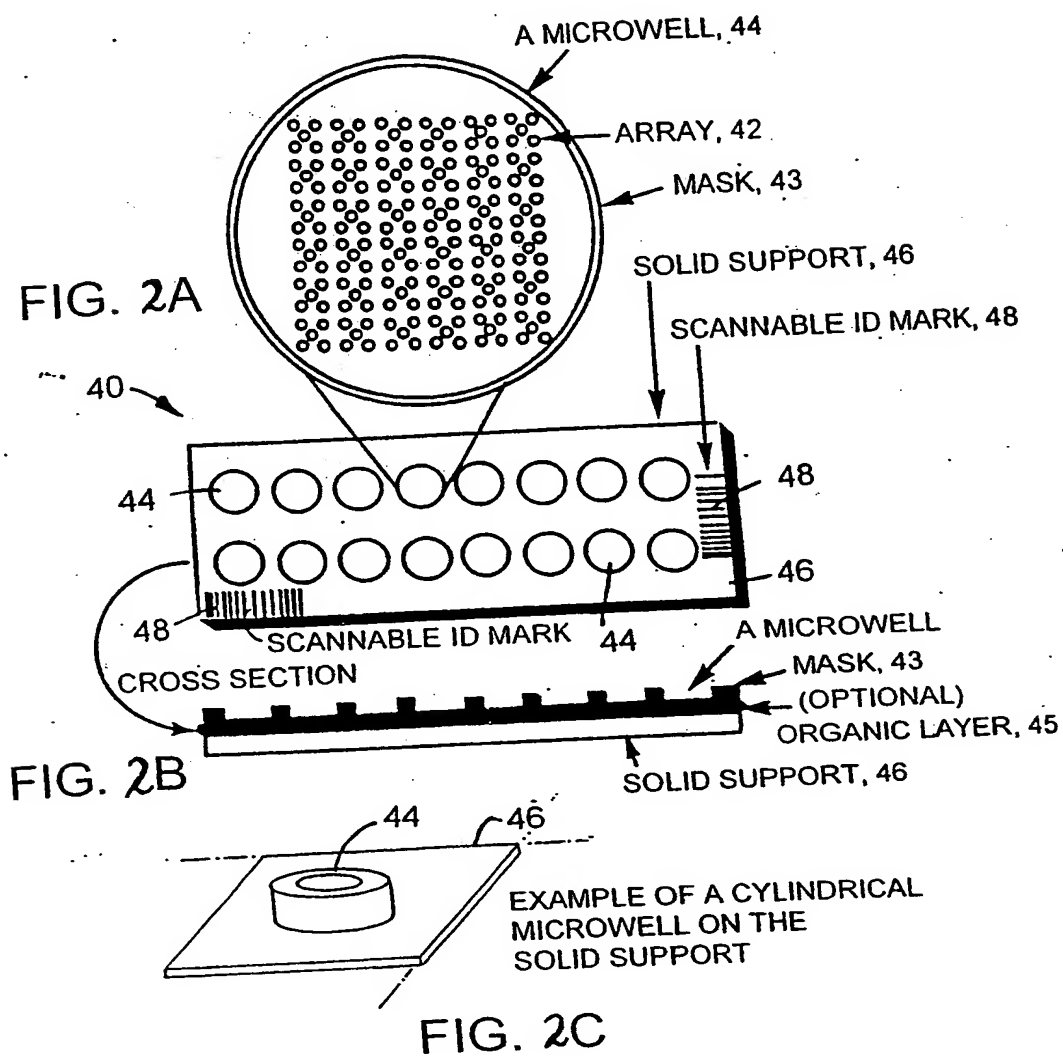
25 32. The device of claim 30, wherein the targets are bacteria or fungi.

33. The device of claim 30, wherein the at least two different types of analytes for each target are a nucleic acid molecule and a surface antigen specific for the target.

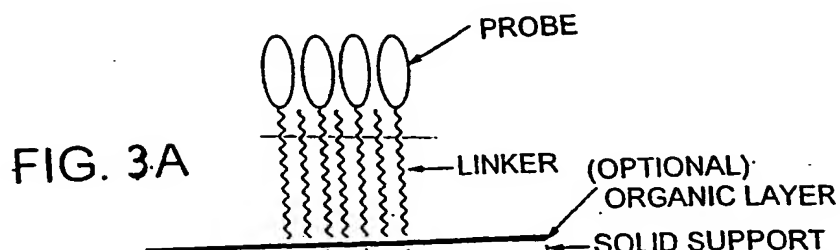
30 34. The device of claim 30, wherein the targets comprise microorganisms and heavy metals.

35. The device of claim 30, wherein the targets are microorganisms listed in Table II.

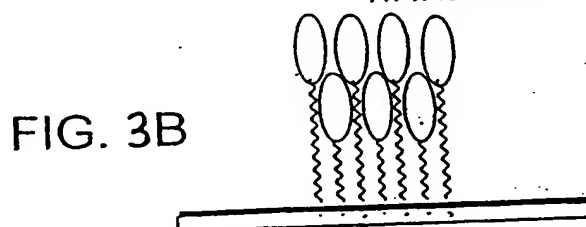








ATTACHING PROBES TO SOLID-SUPPORT WITH LINKERS OF THE SAME LENGTH RESULTS IN LOWER DENSITY OF PROBE ATTACHMENT



ATTACHING PROBES TO SOLID-SUPPORT WITH LINKERS OF DIFFERENT LENGTHS RESULTS IN MUCH HIGHER DENSITY OF PROBE ATTACHMENT

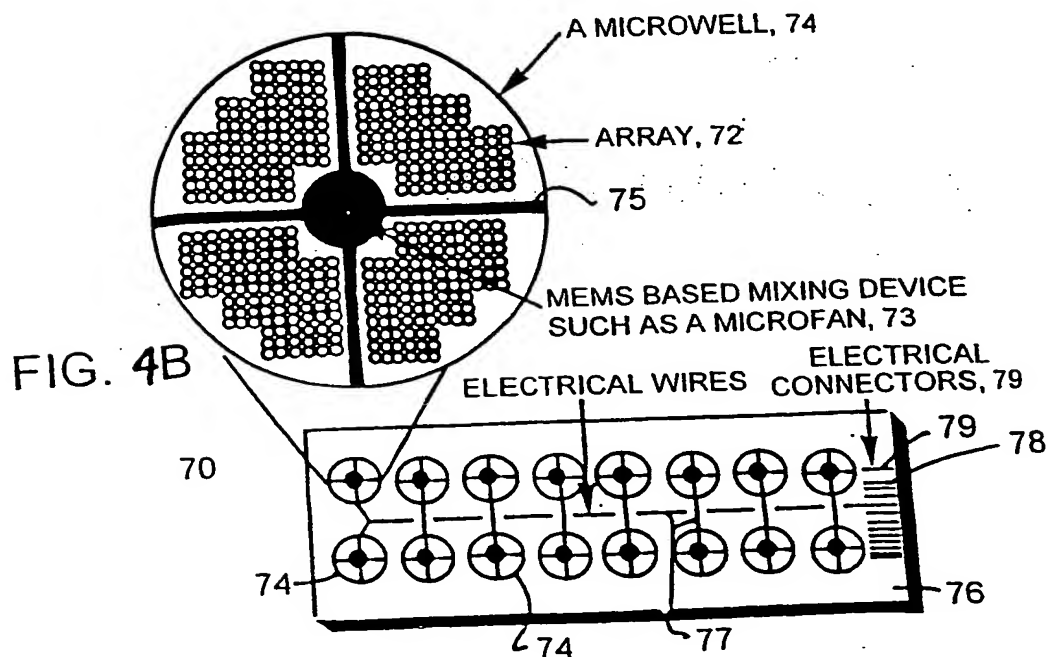
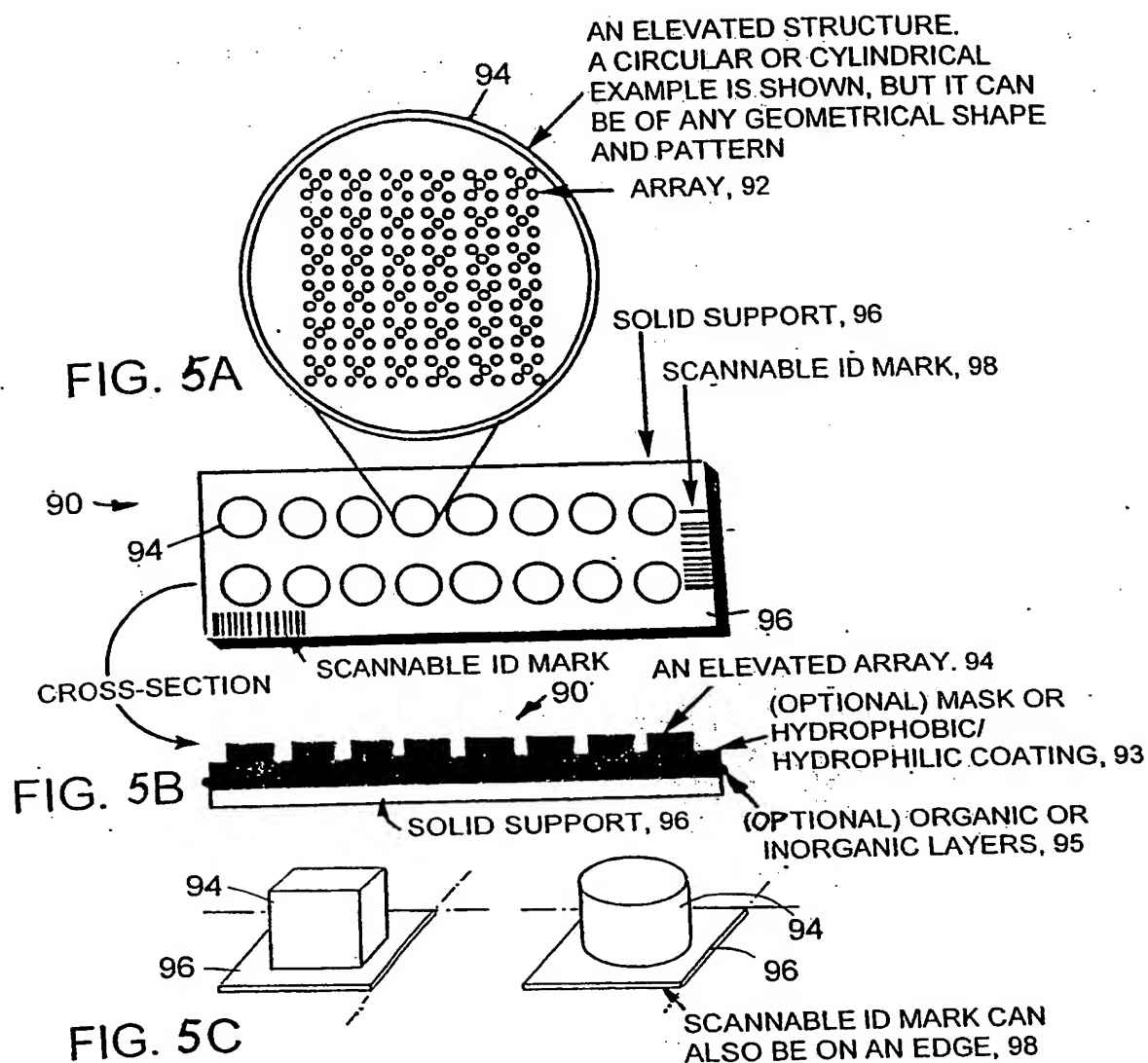
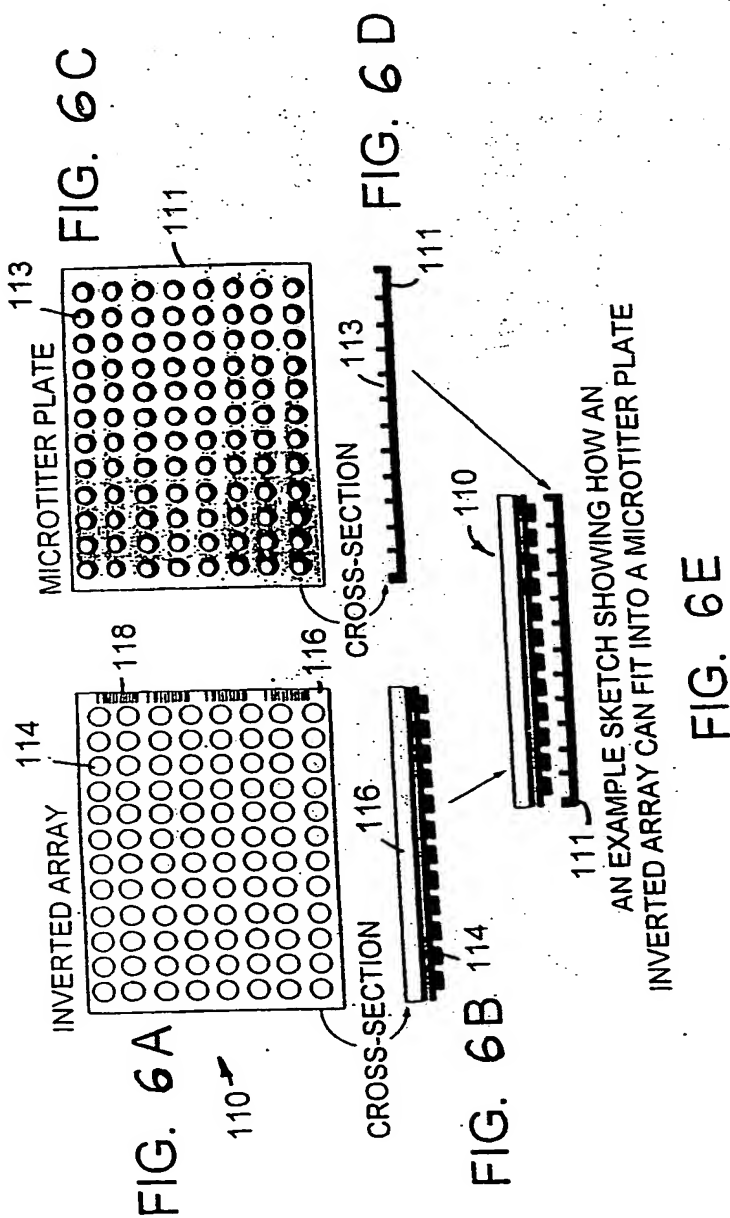
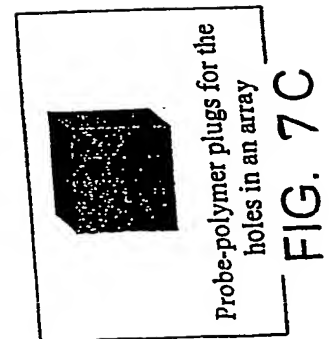
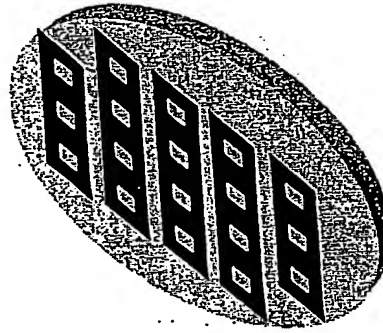
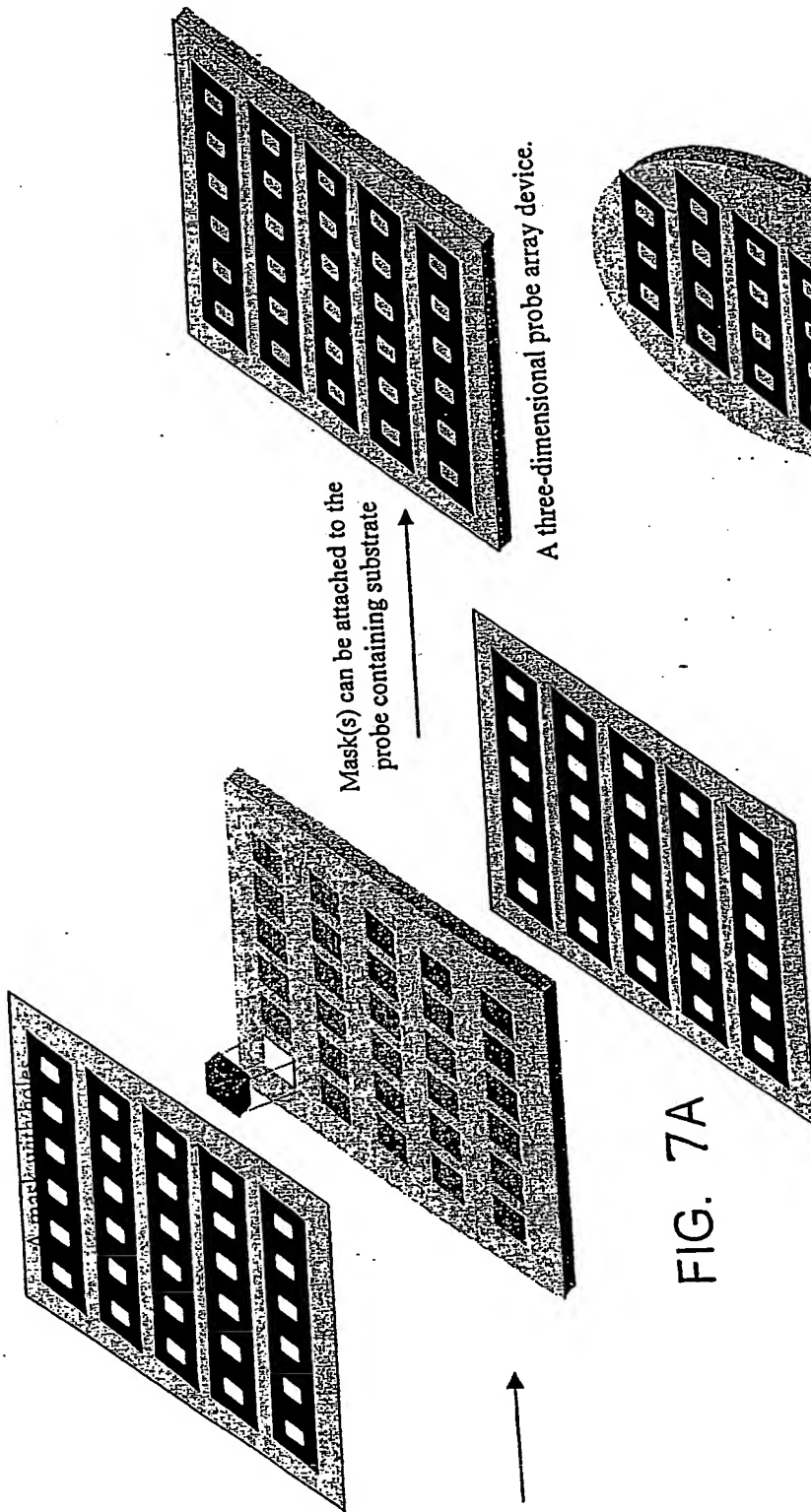


FIG. 4A



EXAMPLES OF ELEVATED STRUCTURES ON SOLID SUPPORT.  
THE STRUCTURES CAN BE CYLINDRICAL OR CUBOID OR ANY  
OTHER GEOMETRICAL SHAPE





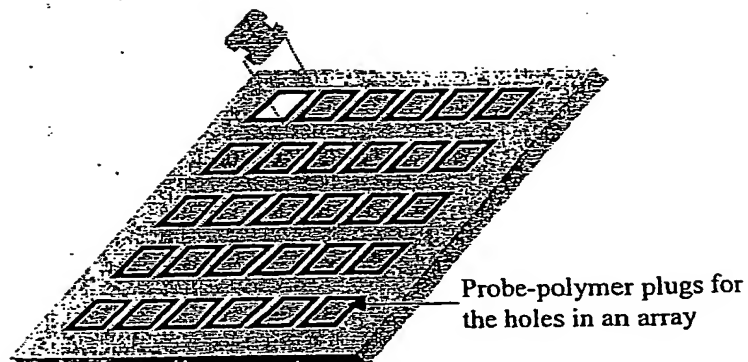


FIG. 8A

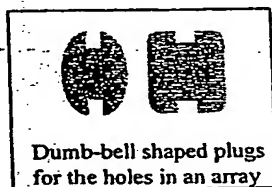
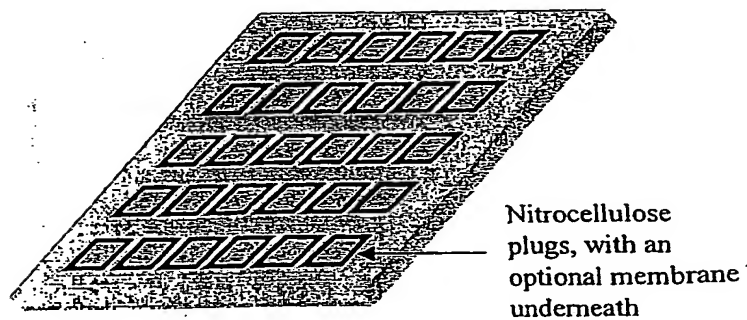


FIG. 8B



Two examples of the types of material that can be used to manufacture the 3D porous array

FIG. 8C

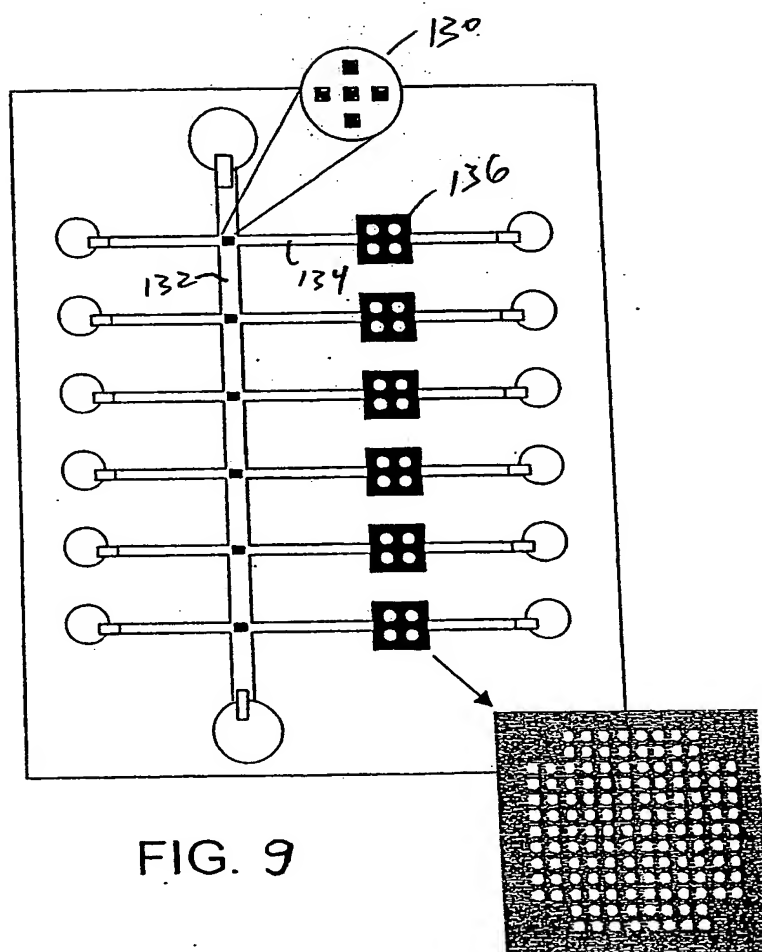



FIG. 9

# Hybridization Enhancement Using Strand-Invader Molecules

Target Double-stranded Nucleic Acid FIG. 10A



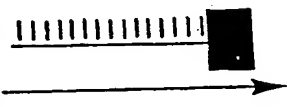
(optional) second oligo  Strand-Invader Probe FIG. 10B



Upon Binding with Strand-Invader  
Target Sequence is Made Single-Stranded  
FIG. 10C

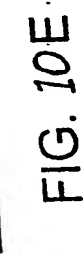


Immobilized Assay Probe  
FIG. 10D



Immobilized Assay Probe Readily Binds  
to the Target Sequence in the Presence of  
Strand-Invader Probe, Forming a much  
more Stable Complex

FIG. 10E  
X = Optional Detector Moiety



A gasket/separator can be used in the current hybridization chambers to place two biochips facing each other in a single chamber for duplicate experiments.



FIG. 11A

FIG. 11B

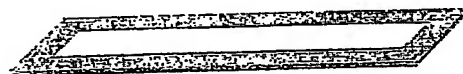


FIG. 11C



Biochip

FIG. 11D



Separator

FIG. 11E



Biochip

FIG. 11F



Holder

A sketch of one example of a new hybridization chamber.  
A hybridization chamber can be devised such that it fits  
two biochips.

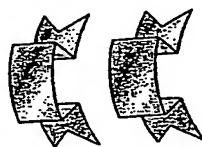
Hybridization Chamber  
Clamps

FIG. 11G



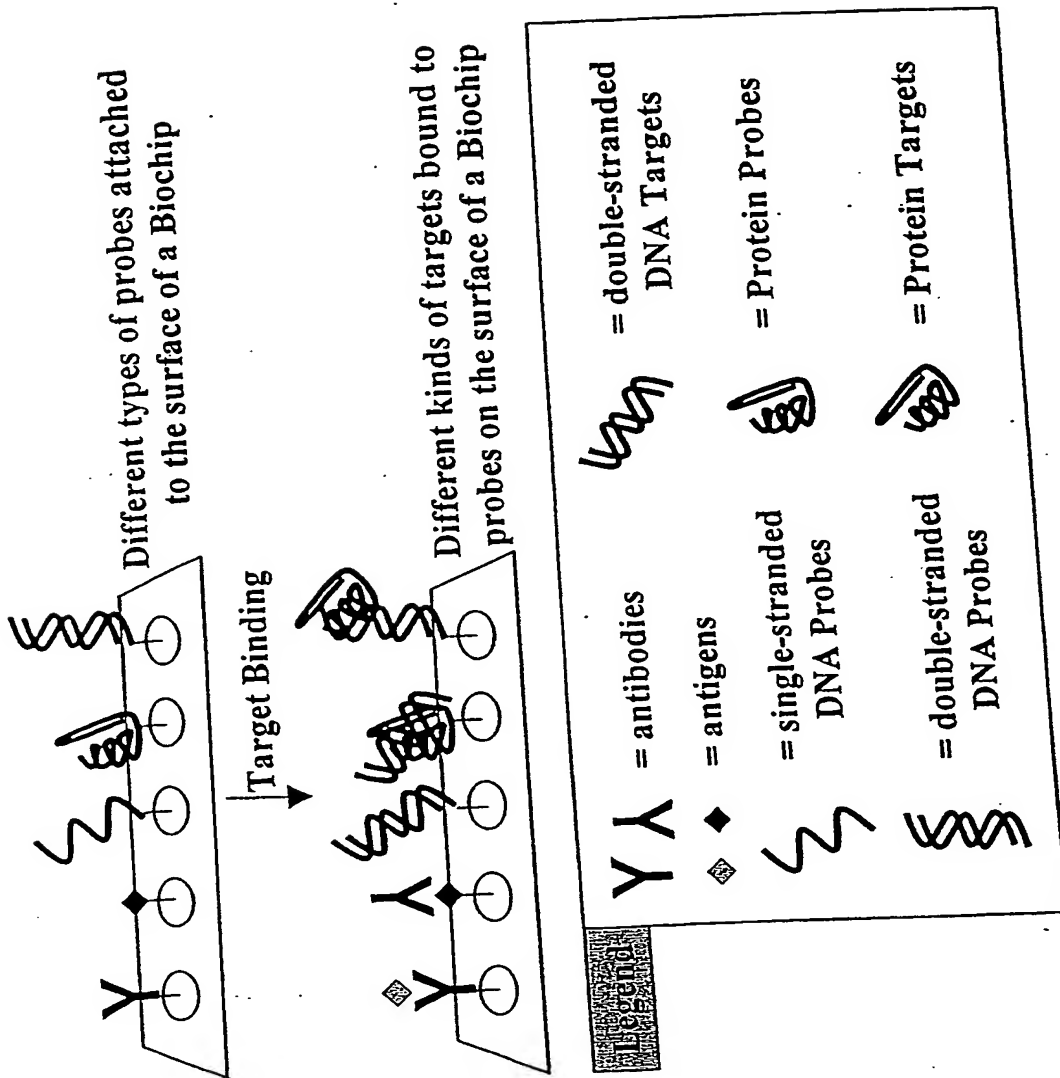


FIG. 12

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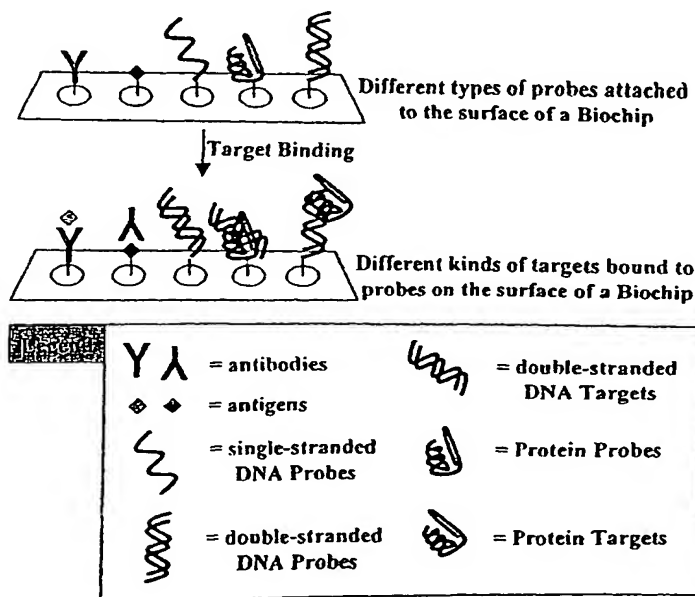
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(57) Abstract: The invention relates to methods and devices for detecting hydrocarbons, such as petroleum, in samples, such as environmental samples, e.g., soil, sand, and water as shown in Figure 12.

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## INTERNATIONAL SEARCH REPORT

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## A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12Q 1/00, 1/68; G01N 33/53, 33/569

US CL : 435/4, 6, 7.1, 7.2, 7.31, 7.32

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## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	PADDA et al. A Novel Gene Encoding a 54 kDa Polypeptide is Essential for Butane Utilization by Pseudomonas sp. IMT37. Microbiology. September 2001, Vol. 147, pages 2479-2491, see entire document, especially figure 3.	1-7, 11, 14, 20-24
X	MCDONALD et al. Methane Oxidation Potential and Preliminary Analysis of Methanotrophs in Blanket Bog Peat Using Molecular Ecology Techniques. FEMS Microbiology Ecology. 1996, Vol. 21, pages 197-211, see entire document, especially figure 4 and table 3.	1-3, 5-6
A	SCHNEEGURT et al. The Application of Molecular Techniques in Environmental Biotechnology for Monitoring Microbial Systems. Biotechnol. Appl. Biochem. 1998, Vol. 27, pages 73-79.	1-35

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## INTERNATIONAL SEARCH REPORT

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### Continuation of B. FIELDS SEARCHED Item 3:

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